X

GENETIC STUDIES WITH KLEBSIELLA

by

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CONTENTS

	Page
Abbreviations	(i)
General Introduction	1
Section I: General Materials and Methods	24
Bacterial strains and bacteriophages	24
Media	24
Amino acids, vitamins and growth factors	27
Drugs and drug sensitivity tests	28
Chemicals	29
Spectrophotometry	30
Centrifugation	30
Membrane filtration	30
Chlorform sterilisation	30
Demonstration of capsules	31
Replica plating	31
Viable counts	31
Mutagen treatment	32
Penicillin selection technique	35
Identification of growth requirements	36
Cross-feeding (syntrophism) tests	37
Isolation of streptomycin resistant mutants	38
Isolation of *lac*$^-$ mutants	38
Isolation of *mal*$^-$ mutants	39
Isolation of non-capsulate mutants	39
Isolation of auxotrophic mutants by penicillin selection	40
Isolation of auxotrophic mutants of *K. aerogenes* strains W52 and W70	41

	Page
Section II: Transformation	43
Introduction	43
Methods	50
Preparation of spheroplasts	50
Preparation of nucleic acid extracts	53
DNA estimations	57
Experiments and Results	58
Attempted transformation of penicillin spheroplasts with nucleic acid obtained by lysis of penicillin spheroplasts	58
Attempted transformation of penicillin spheroplasts using nucleic acid extracted by a number of different procedures	60
Attempted transformation of streptomycin resistance	61
Transformation of cells treated with agents active on cell wall structure	62
EDTA treatment of recipient cells	63
Attempts to obtain "competent" Klebsiella cells	66
Attempted transformation of early log phase cultures of Klebsiella strains	67
Attempted transformation of A3 auxotrophs on the surface of solid media	68
Section III: Transduction	70
Introduction	70
Methods	79
Phage lysates	79
Preparation of indicator plates	79
Phage sensitivity tests	79
Preparation of high titre phage lysates	80

Page

Titration of phages 80

Ultraviolet treatment of lysogenic bacteria 81

Isolation of lysogenic derivatives of sensitive bacteria 81

Adsorption of phage 82

Spot tests for transduction to prototrophy 83

Transduction in broth 83

Transduction to streptomycin resistance 84

Experiments and Results 86

Interactions between K. aerogenes strain A3 and temperate bacteriophages 86

Attempts to detect lysogeny in K. aerogenes strain A3 86

Sensitivity of K. aerogenes strain A3 to phages released by other strains 87

Interaction between K. aerogenes strain A3str^r and strain 8852 89

Interaction between K. aerogenes strain A3 and phage PW52 91

Interaction between K. aerogenes strain A3 and the transducing phages λ and P1kc 92

Interaction between phage D5 and K. aerogenes strain A3 92

Isolation of phages from natural sources 94

Attempted lysogenisation of K. aerogenes strain A3 96

Attempted transduction of K. aerogenes strain A3 to streptomycin resistance 97

Attempted transduction of auxotrophic derivatives of K. aerogenes strain A3 98

Attempted transduction with ultraviolet irradiated phage 99

Attempted transduction of non-capsulate mutants of K. aerogenes strain A3 101

Page

Choice of strains and phages for further experiments 102

Preliminary screening for genetic transfer 104

Induction of phage development in *K. aerogenes* strain W52 105

Isolation and propagation of the phage released by strain W52 106

Attempted transduction with phage PW52 108

Treatment with deoxyribonuclease 109

Centrifugation of a lysate containing phage PW52 110

Frequency of transduction 111

Attempted transduction of non-mucoid mutants of strains W52 and W70 112

Transduction to streptomycin resistance 113

Attempted transduction of a non-mucoid auxotroph of strain W52 114

Transduction of streptomycin resistance and maltose utilisation markers 115

Transduction of nutritional and streptomycin resistance markers 117

Linkage relationships between nutritional markers 119

Linkage relationships between selected nutritional markers 123

Donor phenotype selection tests 127

Section IV: Conjugation 131

Introduction 131

Methods 147

Testing for bacteriocin production 147

Membrane filter mating technique 147

	Page
Experiments and Results	149
Mixed cultivation experiments with Klebsiella auxotrophs	149
Attempted transfer of F-lac to a derivative of K. aerogenes strain A3 in broth	150
Transfer of F-lac to a derivative of K. aerogenes strain A3	151
Attempted transfer of the F factor from E. coli K12 to Klebsiella strains	152
Attempts to detect F-mediated transfer between Klebsiella strains	154
E. coli Hfr x Klebsiella crosses	155
Treatment of a bacteriocinogenic Klebsiella strain with acridine dyes	157
Attempted transfer of the genetic determinant of bacteriocinogeny from K. pneumoniae 1.2 to other Klebsiella strains	158
R-factors - properties of K. aerogenes strain K66	161
Choice of media for selection of drug resistant recipients	162
Transfer of drug resistance to K. aerogenes strain A3str^r	163
Serial transfer of drug resistance between Klebsiella strains	164
Studies on the mechanism of transfer of drug resistance between Klebsiella strains	165
Transfer of multiple drug resistance from K. aerogenes strain K66 to a range of recipient bacteria	167
Transfer of multiple drug resistance from K66 to capsulate and non-capsulate strains	170
Transfer of multiple drug resistance on membrane filters	171

Page

Transfer of multiple drug resistance in HFRT systems 173

Properties of E. coli strain J5 and the R factor which it carries 176

Transfer of the R_{1-14} factor to a Klebsiella recipient 178

Transfer of the R_{1-14} factor from strain J5 to a non-capsulate derivative of strain A3 179

Transfer of the R_{1-14} factor from strain J5 to K. aerogenes strain W107 181

Attempts to detect R mediated chromosomal transfer 184

Sensitivity of bacteria carrying the R_{1-14} factor to male-specific bacteriophage 186

Adsorption of phage MS-2 by bacteria carrying the R_{1-14} factor 187

Incubation of Klebsiella cells carrying the R_{1-14} factor with phage MS-2 189

Discussion 191

Summary 213

Bibliography 218

Acknowledgements 231

ABBREVIATIONS

DNA - deoxyribonucleic acid.

DNase - deoxyribonuclease.

RNase - ribonuclease.

NTG - N-methyl-N'-nitro-N-nitrosoguanidine.

EMS - ethyl methane sulphonate.

2-AP - 2-aminopurine.

EMB - eosin methylene blue.

Ap - ampicillin.

Cp - chloramphenicol.

Tc - tetracycline.

Su - sulphonamide.

Str - streptomycin.

p.f.p./ml. - plaque-forming particles per ml.

GENERAL INTRODUCTION

In bacteria, as in higher organisms, there are two processes which may lead to the formation of new genotypes. The first process involves a heritable change or mutation in the genetic material of a single cell, whereas the second process, which is called recombination, involves the transfer of genetic material from one cell to another. Recombination in bacteria is characterised by a one way transfer of genetic material from donor to recipient cells, and in all cases, deoxyribonucleic acid (DNA) is implicated as providing the genetic specificity of the transferred material.

Three distinct mechanisms of genetic transfer, known as transformation, transduction and conjugation, have been found among bacteria. In transformation, the agent of transfer is naked DNA, extracted from donor cells, and in transduction, bacteriophages act as vectors of genetic material. Both of these processes involve the transfer of relatively small amounts of genetic material, but in certain circumstances the third process, conjugation, may allow larger amounts to be transferred. Conjugation differs from transformation and transduction in that direct cellular contact between donor and recipient bacteria is necessary for genetic transfer. A brief summary of each process will now be presented.

Transformation

Bacterial transformation was discovered by Griffith (1928) in the course of experiments concerned with pneumococcal virulence. Griffith studied the effect of the

injection into mice of living, avirulent organisms simultaneously with virulent organisms that had previously been killed by heat. It had already been known that the virulence of pneumococci depended upon the secretion of a polysaccharide capsule, whose chemical and antigenic specificity was a genetic character of the strain or type of pneumococcus. From his results, it appeared that some substance derived from the heat-killed pneumococci had converted or transformed the living avirulent organisms into virulent ones having the capacity of synthesising the type of capsular polysaccharide characteristic of the heat-killed strain. The agent responsible for this transformation was not identified at the time. However, in 1931 Dawson and Sia succeeded in inducing pneumococcal transformation <u>in vitro</u>, and some years later Avery, MacLeod and McCarty (1944) provided convincing evidence that the transforming agent was in fact DNA.

Further experiments were carried out with different markers, and in 1951 Hotchkiss reported the transformation of penicillin resistance from one strain of pneumococcus to another. Nowadays transformation can be demonstrated for almost any marker whose inheritance by the recipient bacteria is readily detectable (Hayes, 1964). It has also been found that the ability to transform is not a unique attribute of the pneumococcus. Some reports of transformation in other species have not been confirmed or extended (Marmur, Falkow and Mandel, 1963), but reproducible transformation systems have been described in certain species of the following genera:

Haemophilus, Neisseria, Streptococcus, Bacillus and Rhizobium (Spizizen, Reilly and Evans, 1966). Among the enteric bacteria, transformation has been reported in several species (Ravin, 1961), but according to Marmur et al. (1963) the only regularly reproducible system is that of Kaiser and Hogness (1960) for Escherichia coli. In their experiments, Kaiser and Hogness (1960) demonstrated transformation of E. coli K12 for at least two enzymes involved in the utilisation of galactose. The system was rather unusual in that the DNA used for transformation was not extracted from a donor bacterial strain, but from a defective bacteriophage (λdg) which was known to carry the bacterial genes necessary for galactose utilisation. More recently, Taylor and Yanofsky (1964) described a similar system, in which DNA extracted from defective derivatives of phage ϕ80 was used to transform bacterial genes concerned with tryptophan biosynthesis.

Apart from the rather specialised cases in which defective phages are used as the source of DNA, a typical transformation experiment involves treating recipient cells with DNA extracted from donor bacteria. Normally only small fragments of the bacterial chromosome can be transferred in this way, and studies of relationships between markers are therefore restricted to closely linked genes. However, some recent evidence suggests that quite large chromosomal fragments may be transferred when mild procedures are used for DNA extraction, and mapping of extensive linkage groups may well be possible (Kelly and Pritchard, 1965). Earlier

studies have given us a great deal of information about genes determining the biosynthesis of pneumococcal capsules (Jackson, 1962), and about the clustering of genes determining the steps of a biosynthetic process (e.g. Anagnostopolous and Crawford, 1961).

A novel approach to the mapping of the Bacillus subtilis chromosome has been described by Yoshikawa and Sueoka (1963). Based on the hypothesis that chromosomal replication is a polarised process which starts at a fixed point, this approach involves comparing the frequencies of various genetic markers in the DNA of B. subtilis, strain W 23, in exponential and stationary growth phases. The results supported the initial hypothesis, and it was found that at different stages in the growth of a culture there were significant differences in the frequencies with which different markers could be donated by transforming DNA. These differences were thought to correspond to differences in the number of copies of the markers in the DNA and hence in the donor cells. A genetic map was constructed, in which the adenine marker was located near the point of origin from which the chromosome starts replicating, and the methionine and isoleucine markers were located near the terminus. Locations were also suggested for several intermediate markers, and it seems likely that this technique will allow mapping of the broad outlines of the complete replicating unit. Recently, Stonehill and Hutchison (1966) have described a further logical development which suggests that it may even be possible to construct genetic maps without

the usual requirement for a system of genetic transfer. In their study, Stonehill and Hutchison determined the time of duplication of various genes in a synchronous population of bacteria from reductions in the rate of ultraviolet-induced mutation; after the replication of the genes, the rates fell to half their former values. From the resulting data, it was found possible to construct a time-map indicating the sequence of replication of five genes in Streptococcus faecalis. Assuming linear sequential replication of the chromosomal DNA, the time-map presumably reflects the order of the five genes on the chromosome.

Transduction

In 1951, Lederberg, Lederberg, Zinder and Lively found that a lysate of a particular Salmonella strain contained a filterable agent which could transfer bacterial genetic markers to another Salmonella strain. Zinder and Lederberg introduced at that time the term "genetic transduction" to describe not only the phage-mediated transfer that they had observed, but also to describe any process in which genetic fragments (rather than complete genomes) were transferred (Campbell, 1964). However, the word "transduction" is now often used to refer only to the phage mediated process, and this terminology will be adopted here.

In transduction, donor bacteria lyse to release modified phage particles which act as vectors of small fragments of the bacterial chromosome; recipient cells which are infected by

the phages may then incorporate the fragments into their own genetic material. As in transformation, the transferred fragments are small, and genetic analysis by transduction is therefore restricted to bacterial genes which are in close association on the chromosome. Since the original discovery, transduction has been studied in several other groups of the Enterobacteriaceae (Morse, 1954; Lennox, 1955; Coetzee and Sacks, 1960), and in genera such as Bacillus (Stamatin, 1959; Thorne, 1962), Pseudomonas (Loutit, 1958), and Micrococcus (Morse, 1959). Before describing transduction in more detail, it is necessary to discuss briefly some of the properties of bacteriophages.

Bacteriophages are of two types, distinguished according to whether the infected host cell is always lysed (virulent phage) or only sometimes lysed (temperate phage). When a host cell is infected by virulent phage, phage DNA replicates faster than the host chromosome, and controls the synthesis of new phage particles which are released by lysis of the cell. In the case of temperate phage infection, some cells may indeed be lysed, but others may undergo a process known as lysogenisation. When lysogenisation occurs, the host cells survive infection and retain the genetic determinant of phage synthesis in a stable association known as the prophage state. In this state, the phage DNA replicates as a part of the bacterial chromosome, but the genetic determinants of phage synthesis are not expressed. The proportion of cells which become lysogenised depends to a large extent on the

physiological state of the bacterial culture, and can be altered greatly by manipulation of environmental conditions. In a transduction system, the recipient cells must be infected under conditions prejudicial to lysis, but such conditions can often be achieved, even when virulent phages are used.

Most of the known transducing phages will act as vectors in the transfer of any small region of the donor chromosome. This type of transduction is called general transduction, and the first example was discovered by Lederberg et al. (1951). There is another type of transduction, called restricted or localised transduction, in which the activity of transducing phages is strictly limited to a small group of genes closely adjacent to the prophage location on the bacterial chromosome. The first example of restricted transduction was reported by Morse (Morse, 1954; Morse, Lederberg and Lederberg, 1956), and involved phage lambda and the galactose genes of Escherichia coli K 12. A search for a system of restricted transduction can be readily carried out if the site of prophage attachment is known, and in E. coli K 12 such information can be obtained in conjugation studies. Matsushiro (1963) used this approach to discover restricted transduction of the tryptophan genes by ϕ80 in E. coli K 12. However, there is no system available for mapping prophage sites on the Klebsiella chromosome, and so restricted transduction could only be discovered fortuitously.

General transduction

At some stage during phage development, a piece of DNA

from the chromosome of the host bacterium may become incorporated into the phage genome. In general transduction, any phage gene can be incorporated in this way, and incorporation can occur during lytic infection as well as after the induction of lysogenic phage. It appears therefore that replicating (vegetative) phage, and not prophage, is involved in the incorporation process. Now, if a phage which has picked up a fragment of donor chromosome is used to infect an appropriate recipient cell, the fragment of donor chromosome may become stably integrated into the recipient chromosome. The transferred fragment replicates with the recipient chromosome, and a so-called complete transductant results. In certain cases, transferred fragments fail to be integrated, and do not replicate, but the genes they carry can nevertheless express their functional activity. At cell division, therefore, a fragment is transmitted by the cell containing it to only one of the two daughter cells, so that only one cell in a clone possesses the fragment. However, the gene products can be synthesised in each cell which carries the fragment, and their effects may be felt transiently in several generations of daughter cells before they are diluted out by growth. This phenomenon, called abortive transduction, was first demonstrated in connection with motility in Salmonella (Stocker, Zinder and Lederberg, 1953), and was later extended to the study of nutritional markers (Ozeki, 1956). The value of abortive transduction is that it provides a reliable indication of complementation between functionally non-allelic loci.

In general transduction, the probability of any one marker being transferred is low; estimates are usually of the order of 10^{-5} - 10^{-8} per phage particle (Lennox, 1955; Hartman, 1963). Linked transductions, in which two or more markers are transferred simultaneously, are even more infrequent (Stocker et al., 1953), and Lennox (1955) found that the only Escherichia coli characters which he could transduce jointly were those which were known from conjugation studies to be closely linked. Transduction techniques have frequently been used to study the relationships between closely linked genes, and in many cases this implied studying a cluster of genes controlling a single biosynthetic pathway (e.g. Clowes, 1958; Hartman, Loper and Serman, 1960).

Conjugation

In 1946, Lederberg and Tatum observed the formation of prototrophic recombinants in mixtures of different multiple auxotrophic derivatives of E. coli K 12. Recombinant formation was later found to require direct contact between cells of the parent strains, since supernatants or filtrates of cultures of either strain did not yield recombinants when mixed with cells of the other strain (Tatum and Lederberg, 1947). It was also found that no recombinants were formed when cultures of the two parental strains were placed in the separate arms of a U-tube divided by a sintered glass filter which prevented passage of intact cells but allowed thorough mixing of the culture fluids (Davis, 1950).

Although it was not realised at the time, the original E. coli K 12 mating system was heterothallic in nature. It is now believed that the ability to conjugate is conferred upon cells by the presence of discrete genetic elements, which may exist independently of the chromosome and be transferred with high frequency during conjugation (Gross, 1964). The transmissible genetic element, called the sex factor or simply F, is responsible for the donor state and thus for fertility. Donor cells which harbour the F factor independently of the chromosome are known as F^+, and recipient cells which lack the F factor are known as F^-. In an $F^+ \times F^-$ cross, the majority of F^- cells may be rapidly converted to the F^+ state; recombinants inheriting chromosomal genetic determinants may also appear, but with a very low frequency.

From populations of F^+ cells, strains of a new type of donor culture called Hfr (for high frequency of recombination) were isolated (Cavalli-Sforza, 1950; Hayes, 1953). When an Hfr strain is crossed with an F^- strain the yield of recombinants depends on the selected donor marker. With some markers, the yield may be 10^4 or 10^5 fold greater than in F^+ by F^- crosses (Hayes, 1957), while with others the yield may not be much more than in F^+ crosses. The nature of Hfr strains, and of gene transfer during conjugation, became apparent in a brilliant series of experiments on the kinetics of recombinant formation (reviewed in Wollman, Jacob and Hayes, 1956). It was found that the markers of any Hfr strain could be arranged in a continuous gradient with respect

to their frequency of transfer to recombinants. This gradient is thought to be due to the fact that transfer is a linear, oriented process, so that one particular extremity of the chromosome (called the origin) of any one Hfr strain is always the first to enter the recipient cell. Transfer may be interrupted by spontaneous breakage of the chromosome, and as a result recipient cells receive fragments all of which start at the same point but which have variable lengths. Thus unselected markers that are located far from the origin do not appear with significant frequency among recombinants selected for a proximal marker. When distal markers are used for selection, recombinants appear at low frequency; it has been found that the donor state itself behaves as a chromosomal determinant, and is the last marker to be transferred during conjugation.

Further detailed studies by many workers have greatly clarified the role of the sex factor in F^+ and Hfr cells, and Hayes (1966b) has proposed the following model to account for the known facts. The chromosome of Escherichia coli consists of a single DNA duplex which is organised as a continuous loop (Cairns, 1963). The sex factor, F, also appears to consist of a closed loop of DNA, and is approximately 1/50th of the length of the bacterial chromosome. In an F^+ cell, the sex factor replicates independently of the chromosome, but replication seems to be regulated so as to maintain a ratio of one sex factor per chromosome. For conjugation to occur, a cytoplasmic connection between an F^+ (male) cell and an F^- (female)

cell is made. The female remains passive, and so it appears that the cell-to-cell connection is a function determined by the sex factor. The sex factor, or possibly a replica of it, is then transferred to the female, where it initially multiplies rapidly. In this way, females are converted to F^+ cells, which can in turn transfer the F factor to further females. The character of maleness can therefore be spread through the female population with great efficiency. Now, in certain circumstances, genetic interactions can occur between F and the bacterial chromosome. The primary event involves the insertion of the F factor into the chromosome at one or another of a limited number of locations, so that the two structures are now replicated as a single continuous loop of DNA. Insertion is probably due to an act of reciprocal exchange, or recombination, between regions of F and chromosomal DNA which are genetically homologous. When the F factor is inserted, the cell becomes an Hfr donor, and if transfer is initiated it is the whole sex factor/chromosome complex which becomes involved. It has already been mentioned that the Hfr chromosome is transferred as a linear structure, in a particular order for any male strain; Jacob and Wollman (1961) have suggested that it is within the sex factor itself that the chromosome opens up to become a linear structure.

The sex factor can therefore be propagated in two ways: (1) independently in the cytoplasm, in which case it alone is transferred at conjugation, or (2) as an integral part of the

bacterial chromosome, in which case it determines the opening up and transfer of the chromosome as a linear polarised structure. In a further variation, instead of F being inserted into the chromosome, a fragment of the chromosome is inserted into the sex factor. This type of sex factor, which is known as an F-prime (F') factor, also promotes conjugation and its own transfer to female bacteria; the attached bacterial genes are transferred with the F' factor, in the same sequence as they were formerly transferred as part of the chromosome. Furthermore, because these attached genes may be perfectly homologous with the corresponding region of the chromosome of the bacteria which they infect, the F' factor alternates rapidly between insertion into the chromosome in this region, and release to a free, cytoplasmic existence. Thus, populations of male bacteria carrying an F' factor transfer both chromosome and sex factor with great efficiency.

The sex factor can therefore be present or absent in a cell, and can either be independent of or attached to the genome. Jacob and Wollman (1958) proposed that the word "episome" be used to describe elements of this type, and at that time not only the F factor, but also temperate phages and genetic determinants for the synthesis of colicins, appeared to correspond to the definition. Temperate phages do not promote their own transfer from cell to cell by conjugation, and will not therefore be discussed further in this section. Certain determinants of colicin synthesis, on the other hand, resemble the F factor in being able to promote their own

transfer by conjugation (Frédéricq, 1954, 1957), and occasionally chromosomal transfer may also be initiated (Ozeki and Howarth, 1961). However, there has been some controversy about the capacity of colicin factors to assume the integrated state (Clowes, 1963; Driskell-Zamenhof, 1964; Clowes, 1965), and Clowes (1963) has suggested that they should be classified as plasmids rather than as episomes, since the word plasmid denotes all extranuclear structures which are able to reproduce in an autonomous fashion (Lederberg, 1952).

In recent years, a number of other conjugation systems have been described, and many of these are controlled by factors similar to the F factor of Escherichia coli K 12. With some factors, only the properties concerned with the ability to conjugate are known; examples of this type are several factors mediating conjugation between various strains of E. coli (reviewed in Gross, 1964), and the FP factor of Pseudomonas aeruginosa (Holloway, 1955). Other factors have been found to carry additional determinants of properties such as drug resistance (R-factors: Watanabe, 1963a; Datta, 1965), bacteriocin production (P factor in Vibrio cholerae: Bhaskaran, 1960), the ability to utilise sugars (F^{o}-lac factor in Salmonella typhosa: Falkow and Baron, 1962), streptomycin-mutability (in E. coli: Gunderson, Jyssum and Lie, 1962), K-antigen production (in E. coli: Ørskov and Ørskov, 1966), and mucoid antigen production (in E. coli: Hardy and Nell, 1967). Genetic recombination has also been reported in Pseudomonas echinoides (Heumann, 1962) and Serratia marcescens

(Belser and Bunting, 1956). However, there was no evidence of sexual polarity in the Pseudomonas echinoides system, and doubt has been cast on the validity of the Serratia marcescens system by the recent finding of Dushman (1963) that Belser and Bunting's results could be explained without postulating a mating system. Finally in this section, it should be mentioned that the F factor of Escherichia coli K 12 can mediate conjugation between this strain and a number of other strains, including various representatives of Escherichia coli (e.g. strains B and C), Salmonella, Shigella, Serratia marcescens and Vibrio cholerae (Gross, 1964).

Classification of the Klebsiella group

According to the most recent edition of Bergey's Manual (Breed, Murray and Smith, 1957), the genus Klebsiella is a member of the tribe Enterobacteriaceae. In other classification systems, the Klebsiella group is separated from the rest of the tribe Escherichiae, and is accorded generic status in the Klebsiellae tribe (e.g. Kauffmann, 1966).

The precise relationship of organisms of the Klebsiella group to other members of the Enterobacteriaceae is difficult to assess, either from the literature or from classification systems, because much of the necessary information is missing or is confusing. The confusion originated in the very early days of bacteriology, and it is doubtful whether a complete solution has yet appeared. In 1882, an organism called Friedlander's bacillus or Klebsiella pneumoniae was isolated;

three years later a further organism was isolated and called Aerobacter aerogenes. As early as 1929, Edwards called attention to the similarity of Friedlander's bacillus and many cultures then classified as Aerobacter aerogenes. He further suggested that these organisms were so closely related serologically and biochemically that no basis for generic differentiation could be found. This viewpoint was shared to a greater or lesser extent by many subsequent workers, including Parr (1939), Borman, Stuart and Wheeler (1944), Kauffmann (1954) and Edwards and Ewing (1955). However, by the time Bergey's Manual was published in 1957, the situation had not been clarified. The suggestion by various workers that Klebsiella pneumoniae and Aerobacter aerogenes should be combined as Klebsiella pneumoniae was noted, but for stated technical reasons, the two genera were allowed to retain their separate identities. In a discussion of this decision, Breed admitted that no satisfactory method of differentiation had been found, and that identical cultures were classified by some workers as Klebsiella pneumoniae and by others as Aerobacter aerogenes.

A further attempt to rationalise the situation was made by Hormaeche and Edwards (1958). They suggested that the Klebsiella group should be restricted to non-motile organisms, and organisms which were typically motile and could be distinguished from the Klebsiella group biochemically should be placed in the Aerobacter group. Two years later in 1960 the same workers proposed that the name Aerobacter should be

dropped completely, because of the previous indiscriminate application of this name to organisms which should have been called Klebsiella pneumoniae. Hormaeche and Edwards (1960) therefore suggested the adoption of a new genus, to be called Enterobacter, with Enterobacter cloacae as the type species and Enterobacter aerogenes as a species to include the motile organisms previously classified as Aerobacter aerogenes. This system of nomenclature was accepted in 1963 by the Judicial Commission of the International Committee on Bacteriological Nomenclature.

The amalgamation of non-motile organisms previously called Aerobacter aerogenes with Klebsiella pneumoniae in the Klebsiella group caused further problems. With the old nomenclature, organisms isolated from such sources as stools, urine, water and soil were labelled Aerobacter aerogenes, although they were probably culturally and biochemically indistinguishable from Klebsiella pneumoniae. Organisms of the Friedlander's bacillus type could then be called Klebsiella pneumoniae when they were isolated from respiratory infections. The practical distinction which was maintained in this way was useful for the clinical bacteriologist (Darrell and Hurdle, 1964). As a result of the clinical situation, further attempts have been made to distinguish species corresponding to the old Friedlander's bacillus and the old Aerobacter aerogenes within the new Klebsiella group (Cowan, Steel, Shaw and Duguid, 1960; Darrell and Hurdle, 1964). However, confusion is still possible. In 1965, one

group of workers described an organism as a typical Klebsiella pneumoniae serotype 24 (Mahl, Wilson, Fife and Ewing, 1965), while the methods of Cowan et al. (1960) led Steel and Shewan to describe the same organism as Klebsiella aerogenes (Centifanto and Silver, 1964). Division of Klebsiella strains into the two classical types should therefore still be treated with some caution.

It has even been suggested that Klebsiella pneumoniae strains and the old Aerobacter aerogenes-type strains can be distinguished on the basis of their DNA compositions (Mandel and Rownd, 1964). However, Mandel and Rownd reported tests with only two Aerobacter aerogenes strains, and one of these is now labelled Enterobacter aerogenes in the American Type Culture Collection. This organism is presumably motile and might be expected to differ considerably from a typical example of Klebsiella pneumoniae. More recently, Muecke, Bauer and Brock (1966) reported that four Klebsiella pneumoniae and two Aerobacter aerogenes strains showed no apparent difference in DNA base composition. Thus there is only very slight evidence to suggest that the Klebsiella pneumoniae and Aerobacter aerogenes groups are genetically distinct, and it seems likely that genetic interaction between the groups will be possible, if a suitable transfer system can be found.

Another confusing problem relevant to the status of the Klebsiella group is the relationship of Klebsiella strains to E. coli strains. For example, it might be thought that E. coli and organisms formerly classified as Aerobacter

aerogenes would be closely related, since they have long been placed together in the so-called coliform or coli-aerogenes group. Furthermore, biochemical intermediates between Klebsiella (or Aerobacter) and E. coli have frequently been reported (Sanborn, 1944; Heyl, 1957; Kauffmann, 1966), and serological relationships with regard to O and K antigens have been noted (Henriksen, 1954; Ørskov, 1954; Kauffmann, 1966). Other results which suggest a close relationship between these groups have been obtained in comparative studies of the structural and catalytic properties of enzymes (Marmur et al., 1963), in Adansonian analysis (Colwell and Mandel, 1964), and in studies of the transfer of F' factors from E. coli to Klebsiella (de Haan, Stouthamer, Felix and Mol, 1963; Makela, Lederberg and Lederberg, 1962).

However, in 1966 Krieg and Lockhart proposed an arrangement of members of the Enterobacteriaceae based on overall similarities, and the Klebsiella group appeared to justify separation from the Escherichia group into a distinct taxon. Analysis of the base composition of the DNA of E. coli, Klebsiella and Aerobacter strains also suggests a fundamental, genetic difference between the E. coli group and the Klebsiella group as it is now defined. The DNA of E. coli strains generally contains 50-52 per cent guanine plus cytosine, whereas Klebsiella strains generally give results of around 54-58 per cent (Marmur et al., 1963; Mandel and Rownd, 1964; Colwell and Mandel, 1964; Muecke et al., 1966). Falkow (personal communication) has suggested that this difference in

base composition would make it very unlikely that recombinants could be obtained in E. coli x Klebsiella crosses, since the concept that chromosomal genetic recombination can take place only between organisms possessing similar base ratios now seems well established (Baron, 1963).

Systems allowing genetic analysis are available for a number of other groups of the Enterobacteriaceae, including the Salmonella, Shigella and Proteus groups, but the DNA base ratios of representatives of these groups are also significantly different from the base ratios of Klebsiella strains (Marmur et al., 1963). It seems unlikely therefore that chromosomal genetic material derived from any of these groups could be integrated into the genome of a Klebsiella cell, and for this reason it would probably be better to look for intrageneric recombination. Certain of the known sex factors may be able to promote their own conjugal transfer between Klebsiella strains; if the sex factor could become attached to the Klebsiella chromosome, then chromosomal transfer might also occur. The most likely candidate for this role was thought to be a resistance transfer factor, R-100, because the DNA of this factor contains a component whose base composition may be very similar to that of Klebsiella chromosomal DNA. However, it has been found that this R-factor does not integrate in Klebsiella strains, even under conditions favouring integration (S. Falkow, personal communication).

Transformation has been reported to occur in several groups of the Enterobacteriaceae (Ravin, 1961), but according

to Marmur *et al.* (1963) only the defective phage/helper phage systems are regularly reproducible in use. Transducing Klebsiella phages were not available when this study was initiated, and so there was no possibility of using this approach. A rather different approach is suggested by the results of Chargaff, Schulman and Shapiro (1957) and Wacker and Laschet (1960). These authors have reported that *E. coli* cells which have been converted to spheroplasts can act as recipients in transformation experiments, and it seems possible that Klebsiella spheroplasts might also behave in this way.

Transduction mediated by temperate phages has been reported in *Proteus*, *Salmonella*, *Shigella* and *Escherichia* strains. Intergeneric transduction has also been reported, occurring between *E. coli* and *Shigella* (Lennox, 1955), and failing between *E. coli* and *Salmonella* (Zinder, 1963). A search of the literature revealed no indication that any of the known transducing phages had been tested for ability to infect Klebsiella strains; this is a possibility which could be examined. Also, a number of workers have described lysogenic Klebsiella strains (Park, 1956; Ciuca, Eustatziou, Barber, Voinea and Tulpan, 1959; Eustatziou, Eustatziou, Antohi, Horodniceanu, Rusu and Alexandresco, 1962; Clarke, 1964; Milch and Deak, personal communication). Not all phages are capable of transduction, but a considerable proportion are (Hartman, 1963), and some of the temperate phages released by lysogenic Klebsiella strains might well be

transducing phages.

Although a number of workers have attempted to detect genetic transfer in the Klebsiella group, there have been no reports describing clear-cut exchange of chromosomal determinants between Klebsiella strains. One unsuccessful study, which was also perhaps the earliest, was initiated in 1926 (Sherman and Wing, 1937). Clarke (1961) has reported a possible case of transduction in Klebsiella pneumoniae, but this result has not been confirmed. Several other studies will be discussed in later sections.

It has already been mentioned that nomenclature within the Klebsiella group is uncertain, and in the circumstances it is perhaps fortunate that Klebsiella strains can be subdivided serologically (Julianelle, 1926; Edwards and Fife, 1952; Kauffmann, 1966). Several O (somatic) antigen groups are known (Hormaeche, 1958), but the O antigens are masked by the K (capsular) antigens in virtually all freshly isolated strains, and so division into serotypes usually depends upon K antigen determinations. Over seventy capsular serotypes are recognised (Ørskov and Ørskov, 1961), and there are many known strains which do not interact with the standard typing sera (Dr. H. Milch, personal communication). A great deal of information has been obtained about the chemical nature of Klebseilla exopolysaccharides (early work reviewed in Wilkinson, 1958; more recent studies include those of Sandford and Conrad, 1966, and Conrad, Bamburg, Epley and Kindt, 1966), and biosynthetic studies are now in progress

(Sutherland and Wilkinson, unpublished results), but the fact that no suitable system is available has prevented analysis at the genetic level. In the present study, it was intended to look for a system which would make it possible to study genetic aspects of exopolysaccharide production in Klebsiella aerogenes strain A3, since this strain has been intensively studied for a number of years by Wilkinson and his co-workers.

The serological and chemical variety observed in the polysaccharide capsular antigens of Klebsiella strains provides strong justification for an attempt to carry out genetic analysis of such strains. For example, it would be interesting to know how many genes are involved in the biosynthesis of each type of polysaccharide. Preliminary studies of the relevant enzymes (Sutherland, personal communication) suggest that quite a large number of genes would be required, and it may be that a considerable proportion of the genome of a capsulate cell is devoted to a function which does not appear to be essential for growth. This portion of the genome may also vary in size and in nature from strain to strain, depending upon the complexity of the different biosynthetic pathways, unless all of the genes are represented in each strain. In 1960, Jacob, Schaeffer and Wollman tentatively suggested a role for episomes in capsulation phenomena, and, while it is tempting to speculate that a series of supernumerary chromosomes are involved, the necessary evidence will only come when thorough analysis at the genetic level is possible.

SECTION I

GENERAL MATERIALS AND METHODS

Bacterial strains

The bacterial strains used are shown in table nos. 1.1, 1.2 and 1.3.

Bacteriophages

A high titre lysate of phage λ was provided by Dr. I.W. Smith. The male-specific phage MS-2 was provided by Dr. N. Datta, and propagated on S56, an Hfr derivative of E. coli strain K 12. Phage P1kc was provided by Miss K. Smith, and propagated on Shigella dysenteriae strain Sh.

Sterilisation

All basic media were sterilised by autoclaving at 15 lb./sq. in. (121°) for 15 min. Supplements were sterilised separately, either by autoclaving or by membrane filtration, and added aseptically to the basic medium.

Nutrient broth

Nutrient broth was prepared by dissolving 25 g. of Oxoid No. 2 Nutrient Broth powder (Oxoid Ltd., Southwark Bridge Road, London, S.E.1) in 1 l. of distilled water. Unless otherwise stated, the word "broth" refers to this medium.

YE broth

YE broth consisted of nutrient broth enriched with 0.1% (w/v) Oxoid Yeast Extract powder.

TYeCa broth (Meynell and Datta, 1966a)

The basic medium consisted of Oxoid Tryptone 10 g., Oxoid Yeast Extract 1 g., and NaCl 8 g., dissolved in 975 ml. of distilled water. After sterilisation by autoclaving, 15 ml. of a sterile 10% (w/v) glucose solution and 10 ml. of a

Table 1.1

Designation	Characteristics	Obtained from:
A3	*K. aerogenes*, capsular serotype S4	Dr. I.W. Sutherland
A3(0)	non-capsulate mutant of A3	Do.
KP12	Do.	Do.
KP17	Do.	Do.
KP18	Do.	Do.
KP30	Do.	Do.
KP31	Do.	Do.
KP32	Do.	Do.
KP33	Do.	Do.
A1	capsulate	Do.
A4	Do.	Do.
NCTC418	Do.	Do.
1.2(NCTC5054)	*K. pneumoniae*, capsular serotype 1	Dr. C.H. Clarke
1.9	capsular serotype 1	Do.
1.9/R6	uracil requiring mutant of 1.9	Do.
1.9(0)	non-capsulate mutant of 1.9	Do.
1.9(0)/2	adenine requiring mutant of 1.9(0)	Do.
2.1	capsular serotype 2	Do.
2.2	Do.	Do.
2.5	Do.	Do.
2.6	Do.	Do.
2.7	Do.	Do.
2.12	Do.	Do.
2.12(0)	non-capsulate mutant of 2.12	Do.
2.16	capsular serotype 2	Do.
2.22	Do.	Do.
B7380	Do.	Do.
2 Park	Do.	Do.
2 Park.*str*^{r}	streptomycin resistant mutant of 2 Park	Do.
5.6	capsular serotype 5	Do.

Table 1.1 (contd.)

Designation	Characteristics	Obtained from:
5.6 (0)	non-capsulate mutant of 5.6	Dr. C.H. Clarke
54.2	capsular serotype 54	Do.
57.10	capsular serotype 57	Do.
NCTC6869	mucoid colonies on EMB-lactose	Do.
NCTC8167	Do.	Do.
NCTC8172	Do.	Do.
NCTC8808	Do.	Do.
NCTC8821	Do.	Do.
NCTC8843	Do.	Do.
NCTC8852	Do.	Do.
NCTC8866	Do.	Do.
W52	capsulate, serotype unknown	Dr. H. Milch
W53	Do.	Do.
W54	Do.	Do.
W55	Do.	Do.
W56	Do.	Do.
W69	Do.	Do.
W70	Do.	Do.
W71	Do.	Do.
W72	Do.	Do.
K66	probably capsular, serotype 54; carries an R factor	Dr. N. Datta

Klebsiella strains

Table 1.2

Strain	Syn.	Parent	Characteristics	Obtained from
S5	K12	*E.coli* K12	prototroph, *str*^{s}	Dr I.W. Sutherland
S22	AB735	Do.	Hfr, *str*^{s}	Do.
S33	C600	Do.	F^{-} *thr*$^{-}$ *leu*$^{-}$ *thi*$^{-}$ *str*^{r}	Do.
S56	AB312	Do.	Hfr, *lac*$^{-}$ *thi*$^{-}$ *thr*$^{-}$ *leu*$^{-}$ *str*^{r}	Do.
W1655F^{+}	-	Do.	F^{+} *met*$^{-}$ *str*^{s}	Dr I.W. Smith
240	-	Do.	F^{-} *lac*$^{+}$ *met*$^{-}$ *str*^{s}	Do.
242	-	Do.	F^{-} *pro*$^{-}$ *thi*$^{-}$ *lac*$^{-}$ *str*^{r}	Do.
703	-	Do.	prototroph, *str*^{s}	K. Smith
S121	*E.coli* B	*E.coli* B	Do.	Do.
W68	Do.	Do.	Do.	Dr H. Milch
J5R$^{+}_{1-14}$	-	*E.coli* K12	F^{-} *lac*$^{+}$ *pro*$^{-}$ *met*$^{-}$, carries R$^{+}_{1-14}$	Dr N. Datta
G7	-	-	prototroph, *str*^{s}	J. Leach
RVC2907	-	-	Do.	Do.
RVC4925	-	-	Do.	Do.

Escherichia coli strains

Table 1.3

Species	Strain	Obtained from
Shigella dysenteriae	Sh	Dr S.W. Glover
Salmonella typhimurium	LT-2(907)	Dr I.W. Smith
Aerobacter (*Enterobacter*) *cloacae*	NCTC5936	Dr I.W. Sutherland

Miscellaneous bacterial strains

sterile 0.2 M $CaCl_2$ solution were added, so that the final concentration of glucose was 0.15% (w/v) and the final concentration of $CaCl_2$ was 0.002 M.

Nutrient agar

Nutrient agar was prepared by dissolving 15 g. of Oxoid No. 2 Ion Agar in 1 l. of nutrient broth.

Soft agar

Soft agar was prepared by dissolving 5 g. of Oxoid No. 2 Ion Agar in 1 l. of nutrient broth.

Eosin methylene blue (EMB) agar

(1) EMB-lactose was prepared by dissolving 37.5 g. of Oxoid EMB-lactose (Levine) in 1 l. of distilled water. The final concentration of lactose in this medium was 1% (w/v).

(2) EMB-glucose and EMB-maltose were prepared by dissolving 27.5 g. of Difco EMB Agar Base in 900 ml. of distilled water. After sterilisation by autoclaving, the basic medium was supplemented with 100 ml. of a sterile 10% (w/v) solution of the appropriate sugar.

Minimal A

The medium described by Davis and Mingioli (1950) was used, solidified when necessary by the addition of 1.5% Oxoid No. 2 Ion Agar. This medium contained (g./l. medium): K_2HPO_4, 7.0; KH_2PO_4, 3.0; Na_3 citrate.$2H_2O$, 0.5; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0. Glucose solution (10%, w/v) was autoclaved separately and added to the other components after sterilisation to give a final concentration of 0.2% (w/v). Unless otherwise stated, "minimal medium" refers to this medium.

Supplemented minimal media

It was often necessary to supplement minimal media with amino acids or other growth factors. The concentrations suggested by Lederberg (1950) were used, and the various growth factors were sterilised individually (by membrane filtration of aqueous solutions) before being added to the basic minimal medium.

Singly enriched minimal medium (SEM)

This consisted of minimal medium A plus 1.0% (v/v) nutrient broth.

Doubly enriched minimal medium (DEM)

This consisted of minimal medium A plus 10% (v/v) nutrient broth.

M9 minimal lactose medium (Adams, 1959)

This medium contained (g./l. medium): NH_4Cl, 1.0; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; Oxoid No. 2 Ion Agar, 15.0. Lactose (20%, w/v) and $MgSO_4.7H_2O$ (2.7%, w/v) solutions were autoclaved separately and added to the other components after sterilisation to give final concentrations of 0.4% (w/v) and 0.027% (w/v) respectively. M9 minimal maltose medium was prepared in exactly the same way, using maltose instead of lactose.

In the present study, M9 minimal medium was used for selection of mutants or recombinants which utilised a particular carbon and energy source (e.g. lactose or maltose). Minimal medium A was considered unsuitable for this purpose, because it contained sufficient sodium citrate to allow growth

of Klebsiella strains in the absence of any other carbon and energy source.

Tris-Cl medium (Leive, 1965a)

This medium contained 0.12 M tris(hydroxymethyl)aminomethane, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, 2×10^{-3} M K_2HPO_4, 0.25% (v/v) glycerol and 0.5% (w/v) glucose. If necessary the medium was adjusted to pH 7.5.

Tris-maleic buffer (TM buffer: Adelberg, Mandel and Chen, 1965)

The basic medium consisted of (g./l. medium): $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.1; $Ca(NO_3)_2$, 0.005; $FeSO_4.7H_2O$, 0.0025. To prepare TM buffer this basic medium was supplemented with 0.05 M tris(hydroxymethyl)aminomethane and 0.05 M maleic acid, and adjusted to pH 6.0.

Phage buffer (Glover, 1962)

This buffer contained: Na_2HPO_4, 10.5 g.; KH_2PO_4, 4.5 g.; NaCl, 7.5 g.; 0.01 M $MgSO_4$, 15.0 ml.; 0.01 M $CaCl_2$, 15.0 ml.; 1% (w/v) gelatin, 1.5 ml.; distilled water, 1.47 l. The pH was adjusted to a value of 7.2.

Saline

Unless otherwise stated, "saline" refers to 0.85% (w/v) sodium chloride solution.

Amino acids, vitamins and growth factors

The various amino acids, vitamins, purines and pyrimidines listed by Lederberg (1950) were obtained from commercial sources. L-isomers of amino acids were used where possible, but otherwise the DL-compounds proved satisfactory.

Drugs

Streptomycin (Streptomycin Sulphate B.P.) and penicillin (Crystapen Benzylpenicillin B.P.) were obtained from Glaxo Ltd., Greenford, Middlesex. Chloramphenicol, ampicillin and sulphonamides were provided by the Diagnostic Laboratory, Bacteriology Department, University of Edinburgh, and tetracycline (Tetracyn: Pfizer, Ltd., Sandwich, Kent) was provided by Dr. S. McDonald. Concentrated aqueous solutions of streptomycin, penicillin and tetracycline were stored in the frozen state for up to two months; chloramphenicol, ampicillin and sulphonamides were dissolved in liquid minimal medium and used immediately.

Drug-containing media

The basic medium (nutrient agar or minimal A agar) was sterilised by autoclaving and cooled to 60°. Sterile solutions of the appropriate drugs were then added to give final concentrations of 1,000 µg./ml. (streptomycin), 500 µg./ml. (ampicillin or sulphonamide), and 25 µg./ml. (chloramphenicol or tetracycline).

Drug sensitivity tests

Drug sensitivities were normally determined by spreading approximately 1×10^8 bacteria of an overnight broth culture on a nutrient agar plate, and assessing growth inhibition by filter-paper discs containing a drug. However, nutrient agar contains relatively large amounts of sulphonamide inhibitors, and so tests for sulphonamide sensitivity were carried out on minimal A agar. Filter-paper discs impregnated with tetra-

cycline, chloramphenicol, ampicillin and sulphonamides were provided by the Diagnostic Laboratory, Bacteriology Department, University of Edinburgh. In some cases, drug sensitivities were determined by replicating colonies with sterile velvet or with sterile cocktail sticks to media containing the appropriate drug.

Deoxyribonuclease (DNase)

Crystalline DNase was obtained from BDH (British Drug Houses, Ltd., Poole, Dorset). Solutions of the enzyme were prepared in nutrient broth and sterilised by membrane filtration.

Lysozyme

Crystalline egg-white lysozyme (Koch-Light Laboratories, Ltd., Colnbrook, Bucks.) was prepared as a concentrated aqueous solution, sterilised by membrane filtration, and diluted appropriately for use.

Human serum albumin (HSA)

Human serum albumin (Koch-Light) was dissolved in nutrient broth to the required concentration and sterilised by membrane filtration.

Ethylenediaminetetraacetate (EDTA)

The required amount of the disodium salt of ethylene-diaminetetraacetate was dissolved in distilled water or in 5×10^{-2} M Tris-HCl buffer (pH 7.5) and sterilised by membrane filtration.

Antiserum broth

Rabbit antiserum against the non-capsulate organism A3(O)

was provided by Dr. I.W. Sutherland. Immediately before use, a sample of the antiserum was diluted 1/50 in nutrient broth, and the resulting antiserum broth was sterilised by membrane filtration.

Spectrophotometry

A Zeiss Spectrophotometer (model PMQ-2) was operated according to the manufacturers' instructions.

Centrifugation

Bacterial cells in a culture or suspension were deposited by centrifugation for 15-20 min. in an M.S.E. Super Minor bench centrifuge. A dial setting corresponding to c. 2,000 g was used. Bacteriophage particles were deposited by centrifugation at 65,000 g for 60 min. in an M.S.E. High Speed 50 Ultracentrifuge.

Membrane filtration

Millipore membrane filters (Millipore U.K. Ltd., Heron House, 109 Wembley Hill Road, Wembley, Middlesex) of pore size 0.45 μ or 0.22 μ were used throughout, and filtrates were checked for sterility before use. Swinney hypodermic adaptors (Millipore Ltd.) were found to be particularly useful for sterilisation of small volumes (up to 20 ml.) of growth factors, mutagens, and phage lysates. Larger volumes were passed through a 300 ml. Millipore pyrex filter unit.

Chloroform sterilisation

Colonies on a nutrient agar plate were sterilised by placing 0.5 - 1.0 ml. of chloroform on the lid of the plate and leaving the plate inverted for 1-2 hr. at room temperature.

Excess chloroform vapour was then allowed to escape by leaving the plate open for 30 min. at 37°.

Supernatants containing phage, bacteriocin or nucleic acids were shaken vigorously with 0.5 ml. of chloroform per 10-20 ml. of medium. After the chloroform had settled to the bottom the preparation was decanted and aerated until it no longer contained chloroform. Stocks which had been treated in this way were checked for sterility by culture on appropriate media.

Demonstration of capsules

Bacteria were grown on EMB-glucose plates or in broth containing 1% (w/v) glucose, and examined in wet India ink films (Duguid, 1951).

Replica plating

Where agar plates contained 20-100 colonies the growth was replicated to fresh plates with sterile velvet, as described by Lederberg and Lederberg (1952). Velvet suitable for this purpose was obtained from J. Pallu and Lake, 11 Rathbone Place, London, W.1. Occasionally it was found that the sterile velvet technique did not give sufficiently clear-cut results, and in such cases sterile wooden cocktail sticks were applied to individual colonies and then used to inoculate fresh media. This technique allowed approximately 100 colonies to be transferred within 15 min.

Viable counts of bacteria

Viable counts were carried out by the method of Miles and Misra (1938). Serial tenfold dilutions of the bacterial

cultures were prepared in saline. Measured drops (0.02 ml.) of each dilution were then placed on nutrient agar plates and allowed to dry. After overnight incubation at 37°, counts were made in drop areas which contained the largest numbers of colonies without confluence, and the number of viable organisms per ml. was calculated from the mean of at least five counts.

Mutagen treatment - incubation at 45°

A single colony of the strain to be treated was used to inoculate a 10 ml. nutrient broth culture. This culture was incubated at 37° for 16 hr., diluted $1/10^4$ in fresh broth, and reincubated in a water bath at 45° for 48 hr. Samples were then withdrawn, diluted appropriately, and plated on a suitable medium.

Mutagen treatment - ultraviolet irradiation

Overnight broth cultures of the bacteria were washed and resuspended in saline at a density of 5×10^8 cells/ml. Aliquots (4.5 ml.) of the suspensions were then pipetted into 10 cm. petri dishes and exposed to ultraviolet light. Irradiation was normally carried out in the dark at a distance of 25 cm. from the source (an ultraviolet germicidal lamp), and the suspensions were agitated by hand throughout the exposure period. Under these conditions it was found that 0.1 - 1.0% of the bacteria survived treatment for 60 sec.

After irradiation, the various suspensions were mixed with 0.5 ml. portions of X10 broth, and the broth cultures so obtained were incubated at 37° in the dark to avoid photo-

reactivation. Samples were withdrawn at intervals, diluted appropriately, and plated on a suitable selective or indicator medium.

Mutagen treatment - manganous chloride

The method described by Holloway (1955) was used. Centrifuged cells were resuspended in 0.3 M NaCl solution which had been prewarmed to 37°, and incubated at 37° for 60 min. The suspension was centrifuged again, and the cells were resuspended in 0.4% (w/v) $MnCl_2$ solution for 60 min. at 37°. After further centrifugation the cells were resuspended in broth and incubated at 37° overnight to allow segregation and phenotypic expression of the various mutations.

Mutagen treatment - acriflavine

Acriflavine was obtained from British Drug Houses, Ltd., Poole, Dorset. Stock solutions containing 1,000 µg./ml. in water were autoclaved and stored in the dark for up to one week.

An overnight broth culture of the strain to be treated was diluted to c. 10^4 cells/ml. in broth containing 100 µg./ml. of acriflavine and incubated at 37° for 24 or 48 hr. Samples of the culture were then withdrawn, diluted in saline, and plated on a suitable medium.

Mutagen treatment - ethyl methane sulphonate

Ethyl methane sulphonate (EMS) was obtained from Kodak Ltd., Kirkby, Liverpool, and was used as described by Loveless and Howarth (1959). An overnight culture of the parent strain was centrifuged, and the cells were resuspended in an

equal volume of 0.4 M EMS for 15 min. treatment at 37°. After further centrifugation the packed organisms were resuspended in broth and incubated at 37° for 16-18 hr. Samples were then withdrawn, diluted in saline, and plated on a suitable medium.

Mutagen treatment - 2-aminopurine

Measured amounts of 2-aminopurine (obtained from Sigma Chemical Company Ltd., St. Louis, Missouri) were dissolved in broth and sterilised by membrane filtration immediately before use.

Overnight broth cultures of the various parent organisms were diluted to c. 10^4 cells/ml. in broth containing 200 µg./ml. of 2-aminopurine and incubated at 37° for 48 hr. Samples were then withdrawn, diluted in saline, and plated on a suitable medium.

Mutagen treatment - N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

Cells were treated with NTG (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) by the method of Adelberg et al. (1965). Fresh solutions of NTG in sterile distilled water were made for each experiment and used without sterilisation.

An overnight broth culture of the parent strain was diluted with fresh broth to about 1 x 10^6 cells/ml. and incubated at 37° with shaking until the cells were in the logarithmic phase of growth (2-3 hr.). A sample of the culture was then filtered rapidly so that the cells became impinged on a 13 mm. Millipore filter, pore size 0.45 µ. After washing on the filter with 5-10 ml. of TM buffer at

pH 6.0, the cells were suspended by placing the membrane in the original volume of TM buffer (pH 6.0) in a 1 oz. vial and agitating vigorously on a Microid flask shaker. The membrane was removed, and NTG solution was added directly to the suspension to give the desired concentration. In most experiments, the final concentration of NTG was 100 µg./ml., and the final concentration of treated cells was 3-5 x 10^8/ml.

After treatment with NTG for 30 min. at 37°, the cells in a 1 ml. sample of the culture were impinged on a Millipore filter, washed with 5 ml. of cold minimal A medium, and resuspended in 10 ml. of nutrient broth. The culture so obtained was incubated at 37° for 4-5 hr., and then samples were withdrawn and plated on a suitable medium. In some experiments the final cultures were diluted before plating, but this was not always necessary.

Penicillin selection technique

Penicillin is an antibiotic which kills growing bacteria but is innocuous to those which are not engaged in the formation of cell walls. Thus if a mixture of prototrophic and auxotrophic bacteria is well washed and then incubated in minimal medium containing a lethal concentration of penicillin, the prototrophs will be killed as soon as they begin to synthesise cell walls, but the auxotrophs, being unable to grow, will remain viable. The recovery of auxotrophs is not quantitative, but a considerable degree of enrichment can usually be achieved.

Several modifications of the basic technique were tested

in preliminary experiments, and it was eventually found that satisfactory results could be obtained using a method based on the one described by Gorini and Kaufmann (1960). In this method, penicillin treatment was carried out in hypertonic minimal medium to prevent the bursting of spheroplasts (and therefore to minimise cross-feeding of auxotrophs).

A culture of the parent strain was treated with one of the mutagens mentioned above, and then incubated in broth for 6-16 hr. at 37° to allow segregation and phenotypic expression of auxotrophic mutations. After incubation, a 5 ml. aliquot of the culture was centrifuged and washed twice with minimal medium. A small portion of the final pellet was removed with a loop and used to inoculate 5 ml. of minimal A containing 20% (w/v) sucrose and 0.01 M $MgSO_4$ in a 250 ml. Erlenmeyer flask. The flask was shaken vigorously until the population had doubled (1-3 hr.), and then freshly prepared penicillin was added to a final concentration of 3,000 i.u./ml. The mixture was incubated without shaking for a further 2-2½ hr. After this time, the culture was cooled to 0° and centrifuged in the cold room. The packed organisms were then resuspended in minimal medium, and samples were withdrawn for plating on a suitable medium. In most experiments the final suspensions had to be diluted approximately one hundred-fold before plating.

Identification of growth requirements

Auxotrophs isolated following mutagen treatment and penicillin selection were first tested for stability, and any showing a high rate of back mutation to prototrophy were

discarded. Auxotrophs isolated following treatment with NTG were also tested with phage PW52 grown on a prototrophic strain (see later), and any mutants which did not appear to be transducible were discarded. The remaining auxotrophs were then tested for their primary growth requirements by the auxanographic technique (Lederberg, 1950).

An overnight broth culture (10 ml.) of the auxotrophic strain was centrifuged and washed twice in saline. The final pellet was resuspended in 10 ml. of saline, and then a 0.5 ml. sample was withdrawn and added to 10 ml. of molten minimal A agar held at 46°. After mixing by rotation the seeded medium was poured on to the surface of a minimal agar plate, and allowed to solidify. A further five or six plates were prepared in exactly the same way, and each one was dried thoroughly at 37° in an inverted position. Drops of the various amino acid, vitamin and growth factor "pools" described by Lederberg (1950) were then placed on marked areas of the plates and incubated at 37° for 1-3 days. Once a response to a particular pool had been found, the auxotroph was tested against the individual components of that pool.

In the technique described above, the pools and growth factors did not have to be sterile, because growth of surface contaminants could readily be distinguished from sub-surface growth of the auxotroph. Over 40 mutants were tested in this way, and only three or four remained unclassified.

Cross-feeding (syntrophism) tests

Single colonies of two auxotrophic mutants were emulsified

in saline, and streaked parallel to one another (as close as possible without touching) on minimal A agar enriched with 1% (v/v) nutrient broth (SEM). Control streaks of the mutants were made on separate plates. The test and control streaks were compared after incubation at 37^{o} for 2-5 days, and any stimulation of the growth of one mutant by another was noted.

Isolation of streptomycin resistant mutants

In some cases, streptomycin resistant (*str*^{r}) mutants were isolated directly by plating c. 10^{9} cells of the parent organism on nutrient agar containing 1,000 µg./ml. of streptomycin. If this method proved unsuccessful, cultures of the organism were treated with $MnCl_2$ or acriflavine, incubated overnight to allow phenotypic expression, and plated on streptomycin nutrient agar. Any colonies which appeared were purified by serial subculture on streptomycin nutrient agar. The final isolates were then tested for ability to grow on streptomycin-free media, and it was found that none of the *str*^{r} derivatives obtained in the present study were streptomycin dependent.

Isolation of *lac*$^{-}$ mutants of *K. aerogenes* strain A3(0)*his*$^{-}$*str*^{r}

Cultures of strain A3(0)*his*$^{-}$*str*^{r} were treated with various mutagens, and plated on EMB-lactose agar. On this medium, colonies which utilise lactose take up a dense purplish black colouration, often with a green sheen, while colonies which do not utilise the sugar remain of a white or pink colour. In a series of experiments, several *lac*$^{-}$ mutants were obtained, but

only one of them (W60, isolated following treatment with EMS) proved to be relatively stable.

Isolation of *mal*$^-$ mutants of *K. aerogenes* strain W52

Cultures of strain W52 were suspended in TM buffer (pH 6.0), treated with 25 µg./ml. of NTG, washed in minimal medium, and plated on EMB-maltose agar. Several independent experiments were carried out in this way, and 18 white or pale pink colonies were recovered. When these colonies were purified by restreaking on EMB-maltose, it was found that five of them represented *mal*$^-$ mutations.

Two of the *mal*$^-$ mutants proved to be unstable and were immediately discarded. The other three had been isolated independently of one another, and when they were spot-tested with phage PW52 grown on a *mal*$^+$ strain it was found that all three could be transduced to the *mal*$^+$ state.

Isolation of non-capsulate mutants of Klebsiella strains

Cultures of the appropriate parent strain were treated with various mutagens, diluted if necessary, and plated on EMB-glucose agar. The plates were then incubated at 30° (or occasionally at 37°) for 24 hr., and after this time any colonies which differed in appearance from the large, convex, mucoid, parent-type colonies were picked and restreaked on the same medium.

In further experiments, mutagen-treated cultures of strain A3 were washed, resuspended in broth containing A3(0) antiserum, and incubated at 37° for 48 hr. It was noticed that a precipitate formed at the base of most of the tubes.

Table 1.4

Designation	Mutagen	Selection by:	Properties
KP101	45° incubation	A3(0) antiserum	C
KP102	2AP	-	C
KP103	2AP	-	C
KP104	UV	-	C
KP105	2AP	-	NC
KP106	UV	-	C
KP107	Acriflavine	A3(0) antiserum	NC
KP108	2AP	Do.	C
KP109	2AP	Do.	NC
KP110	45° incubation	Do.	C
KP111	2AP	Do.	C
KP112	45° incubation	Do.	C
KP113	UV	Do.	C
KP114	$MnCl_2$	Do.	NC
KP115	UV	Do.	C
KP116	$MnCl_2$	Do.	C
KP117	UV	Do.	C
KP118	$MnCl_2$	Do.	C
KP119	$MnCl_2$	Do.	C
KP120	$MnCl_2$	Do.	NC
KP121	2AP	Do.	C
KP122	2AP	Do.	C
KP123	45° incubation	Do.	C
KP124	2AP	Do.	C
KP125	2AP	Do.	C
KP126	UV	Do.	C
KP127	Acriflavine	Do.	C
KP128	Acriflavine	Do.	C
KP129	45° incubation	Do.	C
KP130	NTG	-	NC

2AP = 2-aminopurine

NTG = N-methyl-N'-nitro-nitrosoguanidine

C = capsulate

NC = non-capsulate

Colony-form mutants of K. aerogenes strain A3

Table 1.5

Designation	Parent strain	Mutagen	Properties
W93	W70	Acriflavine	NC
W94	W52	Acriflavine	NC
W95	W52	UV	NC
W96	W52	2AP	NC
W97	W70	2AP	NC
W98	W70	spontaneous	NC
W99	W70	spontaneous	NC
W100	W52	spontaneous	NC
W101	W52	spontaneous	NC
W102	W93	spontaneous	NC
W103	W52	spontaneous	NC
W104	W93	IV	NC
W106	W70	NTG	C

2AP = 2-aminopurine

C = capsulate

NC = non-capsulate

Colony-form mutants of K. aerogenes strains W52 and W70

Assuming that this precipitate was the result of agglutination of non-capsulate cells by the antiserum, it was hoped that enrichment for non-capsulate mutants might have occurred. Samples were therefore withdrawn from the bottom of each tube (with a fine Pasteur pipette), diluted suitably in saline, and plated on EMB-glucose agar. Once again, any colonies which differed from the parent type were picked and restreaked on the same medium.

Over 50 colony-type mutants of strains A3, W52 and W70 were isolated using the above procedures, and many of them were found to be non-capsulate when examined in wet India ink films. The mutants and their properties are listed in tables 1.4 and 1.5.

Isolation of auxotrophic mutants of Klebsiella strains by penicillin selection

Cultures of the parent strain were treated with one of the mutagens mentioned above, and then exposed to penicillin in minimal A sucrose medium as described by Gorini and Kaufmann (1960). Survivors were plated on doubly enriched minimal (DEM) and incubated at 37° for two days. After this time, colonies which were distinctly smaller than the majority were picked and spread over small areas on nutrient agar plates. These plates were incubated at 37° for 6 hr., and then the growth which had appeared was replicated to minimal agar and nutrient agar with sterile velvet. Any clones which grew on nutrient agar but not on minimal agar were purified by serial subculture, and derivatives which showed a high rate of

reversion to prototrophy were discarded. The others were tested for their growth requirements by the auxanographic technique.

In some experiments the survivors of penicillin treatment were plated directly on nutrient agar, and possible auxotrophs were detected by replicating the resulting colonies to minimal medium.

The various auxotrophs which were obtained are listed in table 1.6.

Isolation of auxotrophic mutants of K. aerogenes strains W52 and W70

Cultures of the appropriate parent strain were treated with 100 µg./ml. of NTG, incubated in broth at 37° for 4-6 hr. to permit the segregation and phenotypic expression of auxotrophic mutations, and plated on nutrient agar. The plates were then incubated at 37° for 24 hr., and after this time any colonies which had appeared were picked with sterile cocktail sticks to nutrient agar and to minimal agar. Isolates showing growth on nutrient agar but not on minimal agar were scored as auxotrophs.

In a typical experiment, 12 independent cultures of strain W70 were treated with NTG. The colonies which appeared on the initial nutrient agar plates were sampled with sterile cocktail sticks, and it was found that 76/150 isolates failed to grow when subcultured to minimal agar. Of these 76 possible auxotrophs, 14 (approximately 3% of the total number of colonies tested) proved to be reasonably stable,

Table 1.6

Designation	Parent strain	Mutagen	Requirement
A3*his*$^-$	A3	UV	histidine
A3/S1	A3	UV	arginine
A3/S2	A3	UV	unidentified
A3/S3	A3	UV	unidentified
A3/S4	A3	UV	proline
W43/A1	W43	UV	arginine
W43/B2	W43	UV	proline
KP129/S1	KP129	UV	unidentified*
KP129/S2	KP129	UV	unidentified*
KP129/S3	KP129	UV	unidentified*
KP105/S1	KP105	UV	unidentified*
KP105/S2	KP105	UV	unidentified*
KP105/S3	KP105	UV	unidentified*
W52/S1	W52	UV	guanine
W52/S2	W52	UV	leucine
W53/S1	W52	UV	unidentified*
W55/S1	W55	UV	unidentified*
W70/A1	W70	2AP	arginine
W71/S1	W71	UV	unidentified*

* No attempt made to identify the primary growth requirement

Auxotrophic mutants isolated following mutagenesis and penicillin selection

transducible, derivatives with single, identifiable growth requirements. The others were either unstable or had unidentifiable (possibly multiple) growth requirements.

It is desirable to guard against the isolation and use of sibling auxotrophic mutants, and so only one derivative was retained from each independent culture, unless of course there were two derivatives with completely different growth requirements. The various auxotrophs which were obtained and identified are listed in table 1.7.

Table 1.7

Derivatives of strain W52			Derivatives of strain W70		
No.	Requirement	Designation	No.	Requirement	Designation
N6	vitamin B1	*thi*-1	L1	proline	*pro*-1
N7	adenine	*ade*-1	L5	threonine	*thr*-2
N14	arginine	*arg*	L8	leucine	*leu*-1
N15	isoleucine + valine	*ilva*-1	L9	isoleucine + valine	*ilva*-2
N21	adenine	*ade*-2	L12	proline	*pro*-2
N22	adenine	*ade*-3	L13	adenine	*ade*-5
N30	methionine	*met*	L14	isoleucine + valine	*ilva*-3
N31	lysine	*lys*	L15	leucine	*leu*-2
N36	cysteine or methionine	*cys.met*	L16	vitamin B1	*thi*-2
N37	adenine	*ade*-4	L17	vitamin B1	*thi*-3
N45	threonine	*thr*-1			
N46	uracil	*ura*			

The mutant numbers given in this table (e.g. *ade*-1, *ade*-2, etc.) refer to the acquisition numbers given to the mutants when isolated.

Auxotrophic mutants isolated following treatment of *K. aerogenes* strains W52 and W70 with NTG

SECTION II

TRANSFORMATION

Introduction

In general terms, transformation may be defined as the integration with the genome of a recipient cell of a small piece of exogenous genetic material, extracted from a donor cell and introduced into the receptor as part of a free DNA particle (Schaeffer, 1964). There are a number of early reports in which transformation of members of the Enterobacteriaceae is described. According to Ravin (1961), reproducible conditions for obtaining transformation were not found in systems involving Shigella paradysenteriae, Salmonella typhimurium, and other Salmonella species, but an apparently reproducible E. coli system has been reported (Boivin, Vandrely and Lehoult, 1945; Boivin, 1947). However, the susceptibility to DNA transforming agents varied widely from one strain of E. coli to another, and unfortunately the E. coli strain in which transformations could be regularly produced has been lost. Two recent E. coli transformation systems (described by Kaiser and Hogness, 1960, and Taylor and Yanofsky, 1964) have already been mentioned, but these systems are unsuitable for application to Klebsiella because they involve a special requirement for defective and helper bacteriophages.

Although negative results may not always be published, there have been reports of unsuccessful attempts to transform E. coli (Atchley, 1951), E. coli and Shigella dysenteriae (Kinoshita, 1955; quoted in Hartman and Goodgal, 1959), and Klebsiella pneumoniae (Balows, Weaver and Humphries, 1955).

In their attempts to detect transformation of K. pneumoniae strains, Balows et al. (1955) carried out an extensive series of in vivo and in vitro experiments. Based on the technique used by Griffith (1928) for pneumococcal transformation, the in vivo studies involved injecting mice with a mixture of killed capsulate cells and living non-capsulate (S) cells. In no case was transformation to capsulation observed. Further experiments were therefore carried out using an in vitro system. Potentially transforming DNA was extracted from four different capsulate donor strains by a variety of techniques, and in some cases the extract was obtained from cells which had previously been decapsulated with a phage enzyme. The extracts were then applied to non-capsulate (rough or smooth) recipient cells, which were growing in environments thought to be conducive to transformation. For example, bovine albumin was added to some mixtures, and polyvalent S antiserum was added to others. Tests were carried out using as potential recipients twenty different non-capsulate strains and one normally capsulate strain which had been decapsulated with phage enzyme. Type transformation was never observed, although the authors point out that many of the techniques which they used had proved successful with other organisms.

The reasons for failure in experiments of this type are not easy to assess, particularly when the techniques used are not described in any detail. Thus the authors' conclusion that none of their strains furnished "competent" cells may

well be valid if not very informative. However, it appears that the only genetic markers tested were those concerned with capsulation. Selection for a few capsulate cells in a large population of non-capsulate cells is notoriously difficult, and it is therefore possible that rare capsulate transformants would have been missed, particularly in experiments where no apparent attempt was made to provide a selective medium. In contrast to capsulation markers, drug resistance and nutritional markers allow the application of strong selective pressures, and it might be wise to use such markers in experiments designed to screen for transformation.

This suggestion is supported by the findings of Chargaff, Schulman and Shapiro (1957) and Wacker and Laschet (1960). Chargaff *et al.* (1957) applied DNA-containing material extracted from cells of a wild type strain of *E. coli* to penicillin "protoplasts" of a lysine-requiring mutant strain. Incubation with the DNA-containing material increased the proportion of prototrophs about five-fold in the absence and twenty-fold in the presence of human serum albumin. Three years later, Wacker and Laschet (1960) carried out a series of experiments using a modification of the procedure of Chargaff *et al.* (1957), and it was claimed that *E. coli* "protoplasts" could be transformed with respect to vitamin B12 synthesis and streptomycin sensitivity. Both sets of results suggest the occurrence of a process resembling transformation, but a number of people have drawn attention to the fact that certain vital controls were not carried out. The work of Chargaff

et al. (1957) has been criticised because no results of control experiments with DNA preparations from the mutant itself were reported, and the sensitivity of the system to DNase was not determined (Ravin, 1958; McQuillen, 1960). Wacker and Laschet (1960) checked the sensitivity of their transforming principle to DNase, but again it appears that no control was performed to check the specificity of the DNA (Ravin, 1961). Furthermore, McQuillen (1960) has suggested that the results of Chargaff et al. (1957) can be explained in terms of differential protection of prototrophs in penicillin media, and it may not therefore be necessary to postulate a specific transformation. In view of these criticisms, and particularly because several other investigators (including Lederberg and St. Clair, 1958) have failed to transform E. coli "protoplasts", it seems that the issue must be regarded as unsettled.

It may well be possible to extract genetically active DNA from almost any bacterial strain, and if such a preparation is applied to a recipient strain whose genome is largely homologous with that of the donor strain, one might expect transformation to occur. However, only certain strains of species known to be transformable possess the properties which allow them to act as recipients (Marmur et al., 1963). For example, if a strain secretes an active extracellular DNase which depolymerises homologous as well as heterologous DNA, it may be impossible to transform such a strain unless the enzyme can be inactivated without inactivating the potentially transforming

DNA (Ravin, 1961). This problem may not arise in the present study, because Rothberg and Swartz (1965) have shown that none of the 103 Klebsiella/Aerobacter strains which they tested produced an extracellular DNase. A problem which is more likely to be relevant to studies with Klebsiella is suggested by the finding of Ravin (1957) that transformability of the pneumococcus is related inversely to the amount of capsule secreted, but any difficulty of this nature can probably be overcome if non-capsulate Klebsiella mutants are used as recipients.

The investigation of the physiological conditions contributing to the transformability of the pneumococcus was begun in 1946 by McCarty, Taylor and Avery, and from this and subsequent work the notion of competence has emerged. Competence may be defined as the transient physiological state in which bacteria must be at the time of exposure to DNA if transformation is to follow (Schaeffer, 1964). The factors which determine competence may vary from species to species, and the precise influence of these factors is not always understood. However, two main theories have been proposed to explain the ability of certain bacteria to take up DNA irreversibly (Ravin, 1961; Hayes, 1964). Firstly, the competent bacterium may be regarded as one having attained an increased permeability to large molecules, resulting from discontinuities in the cell wall (Hayes, 1964). This hypothesis is often termed the "localised protoplast" hypothesis (Ravin, 1961), and in this case the factors which determine competence are presumably

related to the appearance of cell wall discontinuities. Thus the practice of using E. coli spheroplasts or "protoplasts" as recipients (Chargaff et al., 1957) may provide a method of preparing competent cells. However, the second hypothesis suggests that competence is determined by the synthesis of enzymatically active receptor sites at the bacterial surface (Hotchkiss, 1954; Spizizen, Reilly and Evans, 1966), and if this is the case the spheroplast technique may merely provide a method of bypassing competence. Similarly, the requirement for helper, non-defective, bacteriophages in the λdg and Ø80-try transformation systems may also reflect a situation in which competence is bypassed. Ravin (1961) has brought the two hypotheses together, by suggesting that the receptor sites may be on the bacterial membrane, in which case access may be restricted when a cell wall surrounds the membrane. The ability of DNA to reach the receptor sites may therefore depend on the formation of a "localised protoplast".

Support for the idea that there is a specific receptor for DNA has been obtained recently by a number of workers. For example, in Streptococcus and Diplococcus systems it has been shown that the supernatant of a competent culture is able to confer competence on an incompetent culture of the same strain (Pakula and Walczak, 1963; Pakula, 1965; Tomasz and Hotchkiss, 1964; Tomasz, 1965). The factor responsible for this conversion is probably a protein (Pakula and Walczak, 1963; Tomasz, 1965), and in the pneumococcal system Tomasz and Mosser (1966) found that the competence factor appeared to

react with some structure at the cell surface. As a result of this, the cells acquired the ability to bind DNA molecules. It seems possible, therefore, that one important aspect of competence involves the attachment of DNA to a specific protein at a fixed receptor site.

Preparation of Spheroplasts

McQuillen (1960) has defined spheroplasts as "globular forms in which cell wall structure has been modified (e.g. by growth in penicillin) rather than totally removed". Several of the authors cited in this section use the word "protoplast" with quotation marks to describe similar forms, but because it may not be possible to prepare true protoplasts of gram negative bacteria (McQuillen, 1960), it seems more logical to use the word spheroplast. In some cases, a prefix will be used to indicate the method of induction of the modified globular forms (e.g. penicillin spheroplast, lysozyme/EDTA spheroplast).

From several well established techniques for preparing spheroplasts, three were chosen for use. They are:

(1) a penicillin technique, chosen because Chargaff et al. (1957) and Wacker and Laschet (1960) used this successfully in transformation of E. coli;

(2) a lysozyme/EDTA technique, based on the work of Sutherland (1958); and

(3) a freezing/thawing/lysozyme technique (Kohn, 1960).

The second two methods were included to broaden the range of cell wall treatments, especially since penicillin spheroplasts of Klebsiella may differ in some respects from E. coli spheroplasts. Other possible methods of preparing spheroplasts were not attempted, for a number of reasons. For example, the diaminopimelic acid deprivation technique (McQuillen, 1958)

requires a DAP^- mutant, and might therefore involve lengthy isolation procedures.

1. Penicillin spheroplasts. The procedure used for the preparation of penicillin spheroplasts follows closely the description of Chargaff et al. (1957). The K. aerogenes strain to be used as a recipient was grown overnight at 37° in Penassay broth (Difco), and a 3 ml. portion of the culture was added to a 10 ml. portion of Penassay broth containing 20% (w/v) sucrose, 0.2% (w/v) $MgSO_4.7H_2O$ and 1,000 units/ml. of benzylpenicillin. The mixture was shaken at room temperature for 1 hr., and then at 37° for 5 hr., by which time conversion to spheroplasts appeared to be complete. Chargaff et al. (1957) found that conversion was complete after 2½-3 hr., but examination by phase contrast microscopy showed that up to 6 hr. was required in the present study. In attempts to repeat the transformation technique of Chargaff et al. (1957), 1% (w/v) human serum albumin (Koch-Light) was added to certain aliquots of the penicillin medium.

2. Lysozyme/EDTA spheroplasts (Sutherland, 1958). Recipient cells at an optical density of about 0.6 (at 540 mμ.) were suspended in 0.5 M sucrose containing 0.1% (w/v) $MgSO_4.7H_2O$ and 5×10^{-2} M Tris-HCl buffer (pH 7.5); egg-white lysozyme (final concentration: 33 μg./ml.) and EDTA (final concentration: 300 μg./ml.) were then added, and the suspension was incubated at 37° for 45 min. Conversion to spheroplasts was

determined by phase contrast examination of wet films. It was found that there was less than 5% conversion, even when incubation in the presence of lysozyme and EDTA was prolonged for 6 hr. A control preparation of cells suspended in Tris-HCl buffer (pH 7.5) containing only sucrose (0.5 M) and $MgSO_4.7H_2O$ (1% w/v) was then examined, and it was found that 3-5% of the cells had been converted to globular spheroplast forms after 6 hr. incubation at 37°. It seems possible, therefore, that in both cases the cell wall damage was caused by autolysis, and that lysozyme had little if any effect when it was present. Support for this suggestion can be found in the literature. Mitchell and Moyle (1956) reported the formation of "protoplasts" of Bacterium coli and Aerobacter aerogenes in a medium consisting of sodium malonate (0.3 M, pH 7.0) and L-arabinose (0.5 molal), and they attributed the cell wall damage to autolysis. A similar mechanism was proposed to explain spheroplast formation of E. coli in 0.4 M sodium acetate buffer (pH 6.0) stabilised with 1.6 M sucrose, but although this system had the characteristics of an enzymic process (pH optima, temperature, effect of -SH group inhibitors, etc.), attempts to isolate autolytic enzymes were unsuccessful (Mohan, Kronish, Pianotti, Lepstein and Schwartz, 1965).

3. Freezing/thawing/lysozyme spheroplasts (Kohn, 1960). Exponential cells of the recipient culture were harvested by centrifugation, and resuspended in 20% (w/v) sucrose broth

containing 0.2% (w/v) $MgSO_4.7H_2O$. Lysozyme was added 10-15 min. later to a final concentration of 30 µg./ml. After a further 2 min., the mixtures were rapidly frozen in an acetone-dry ice bath. The frozen samples were then thawed by immersion in a 37° water bath. Examination by phase contrast 30 min. after thawing indicated that over 90% of the cells had been converted to spheroplasts.

Results

By phase contrast microscopy, it was noted that conversion to spheroplasts was complete using method (1), and virtually complete using method (3), but very few spheres (or rods with distended portions) were observed using method (2). This last finding is in agreement with Gebicki and James (1958) for Aerobacter aerogenes, and Hamilton-Miller (1966) for three strains of Klebsiella aerogenes, although Sutherland (1958) has obtained lysozyme/EDTA spheroplasts of Aerobacter cloacae strain 5920. However, cells treated with lysozyme and EDTA were included in some transformation experiments, in the hope that these agents might cause minimal structural damage and yet have sufficient effect to facilitate entrance of DNA.

Preparation of Nucleic Acid Extracts

1. Detergent lysis (Marmur, 1961). All solutions and solvents used in this procedure were prepared as described by

Marmur (1961). The ribonuclease was obtained from BDH, and a 0,2% solution in 0.15 M NaCl (pH 5.0) was heated at 80° for 10 min. to inactivate any contaminating DNase. Concentrated saline/citrate (1.5 M NaCl; 0.15 M sodium citrate) was used to adjust dilute saline/citrate (0.015 M NaCl; 0.0015 M sodium citrate) to the concentration of standard saline/citrate (0.15 M NaCl; 0.015 M sodium citrate).

Donor bacteria grown to the logarithmic phase of their cycle (2-3 g. wet packed cells) were harvested and washed with saline/EDTA (0.15 M NaCl plus 0.1 M EDTA, pH 8.0). After collection by centrifugation, the cells were resuspended in a total volume of 25 ml. of saline/EDTA. Lysis was effected by the addition of 2.0 ml. of 25% (w/v) sodium lauryl sulphate, and the mixture was placed in a water bath at 60° for 10 min. After cooling to room temperature, sodium perchlorate was added to a final concentration of 1 M, and the mixture was shaken with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) for 30 min. The resulting emulsion was separated into three layers by a 5 min. centrifugation at 8,000 g, and the upper aqueous layer was removed. The nucleic acids were precipitated from this layer with 2 volumes of ethanol, and the resulting threadlike precipitate was transferred to dilute saline/citrate. Sufficient concentrated saline/citrate was then added to adjust the concentration to that of standard saline/citrate. Deproteinisation with chloroform/isoamyl alcohol was repeated, until very little protein was seen at the interface. Nucleic acid was precipitated from the

supernate obtained in the last deproteinisation step, dispersed in saline citrate, and treated with 50 µg./ml. ribonuclease. A further two deproteinisation steps were carried out, and the final aqueous layer was again treated with ethanol. The precipitated nucleic acid was dissolved in 9.0 ml. of dilute saline/citrate, and then 1 ml. of acetate/EDTA was added. With rapid stirring, 0.54 volumes of isopropanol were added, and the DNA which precipitated in a fibrous form was collected. After washing free of acetate and salt (in ethanol), the DNA was sterilised by exposure to 75% ethanol for several hours.

2. Modified Marmur's method (Gwinn and Thorne, 1964). The above procedure was repeated, but was finished after one deproteinisation and one ethanol precipitation. The precipitate was dissolved in 2 M NaCl, and was not purified any further before use.

3. Ultrasonication of normal cells (Balassa, 1963). Cooled overnight cultures of the appropriate donor strains were partially disintegrated in an MSE 60 watt ultrasonic disintegrator, and the crude extracts so obtained were sterilised by treatment with a small volume of chloroform. Each extract was tested for sterility before use in a transformation experiment.

4. Penicillin lysis of normal cells (Balassa, 1963). Overnight

broth cultures of the donor strains were lysed with 1,000 units/ml. of penicillin, and an excess of penicillinase (Wellcome) was then added to neutralise residual penicillin. The crude extract was sterilised by the chloroform method and checked for sterility when used.

5. Lysis of freezing/thawing/lysozyme spheroplasts (Kohn, 1960). Spheroplasts were prepared by Kohn's technique, and shocked by suspension in 0.85% (w/v) NaCl. The bacterial debris was removed by centrifugation, and the supernate was sterilised by the chloroform method before use as a nucleic acid extract.

6. Lysis of penicillin spheroplasts (Chargaff et al., 1957). A 300 ml. culture of the donor strain was converted to penicillin spheroplasts by the technique of Chargaff et al. (1957). Because conversion to spheroplasts was not complete after 3 hr., incubation was prolonged for up to 6 hr. After washing the spheroplasts in 20% (w/v) sucrose, crude nucleic acid was extracted by repeated treatment with cold 0.1 M NaCl (containing 0.05 M sodium citrate) followed by treatment with 10% (w/v) NaCl. The extraction mixtures were centrifuged at 23,000 g, and nucleic acid was precipitated from the supernates with 95% ethanol. The detailed description of Chargaff et al. (1957) was followed throughout the whole procedure, the only deviation being the time of incubation in penicillin medium.

DNA estimations. DNA estimations were carried out by the method of Burton (1956), using commercial DNA from herring sperm as a standard. Readings were carried out at 600 mμ. on a Zeiss PMQ2 spectrophotometer.

Experiments and Results

Attempted transformation of penicillin spheroplasts with nucleic acid obtained by lysis of penicillin spheroplasts

A preliminary attempt was made to detect transformation of penicillin spheroplasts of W2 (a non-capsulate, *his*$^-$ mutant of *K. aerogenes* A3), using nucleic acid extracted from the prototrophic strain A3(0).

Crude DNA was extracted from the donor, A3(0), by the lysis of penicillin spheroplasts (Chargaff *et al.*, 1957). Penicillin spheroplasts of the recipient auxotroph were prepared by the technique of Chargaff *et al.* (1957), with and without 1% (w/v) human serum albumin, and in the presence and absence of sufficient nucleic acid from the prototroph to give a final concentration of approximately 2 µg. DNA per ml. When conversion to spheroplasts seemed complete, the preparations were washed with cold 20% (w/v) sucrose ± albumin, and then shaken for 30 min. at 37° in 20% (w/v) sucrose ± albumin ± nucleic acid. Next, each mixture was added to an equal volume of double strength Penassay broth containing 20% (w/v) sucrose ± albumin ± nucleic acid. Gentle shaking at 37° for 2 hr. sufficed to allow the conversion of the cultures to rod-shaped forms; by this time the cells were relatively stable and could be shaken more vigorously in the same medium. After 14 hr. vigorous shaking, 10 ml. aliquots of each culture were removed, washed three times with 0.15 M NaCl, and resuspended in 2 ml. saline. Suitable dilutions of the suspension were then spread on plates of minimal A with and without histidine.

Table 2.1

Additions:		No. of organisms/ml. on:	
Crude DNA from prototroph	Albumin	Min. A + *his*	Min. A
-	-	2×10^9	17
-	+	3×10^9	8
+	+	1×10^9	11
+	-	1×10^9	14

Attempted transformation of strain A3(0)*his*$^-$ with nucleic acid extracted from strain A3(0)

Results

The results indicate that incubation of strain A3(0)*his*$^-$ with nucleic acid ($\pm$ human serum albumin) did not lead to the detection of more prototrophs than were found in the absence of nucleic acid.

Attempted transformation of penicillin spheroplasts using nucleic acid extracted by a number of different procedures

The results of the last experiment were negative, and it was felt that a number of other methods of preparing the nucleic acid extract should be attempted. Nucleic acid was therefore extracted from cultures of *K. aerogenes* strain A3(0) by the following methods:

(1) detergent lysis of normal cells, followed by extensive purification of DNA (Marmur, 1961);
(2) detergent lysis of normal cells (Gwinn and Thorne, 1964);
(3) ultrasonic treatment of normal cells (Balassa, 1963);
(4) penicillin lysis of normal cells (Balassa, 1963);
(5) freezing and thawing of normal cells and lysozyme treatment (Kohn, 1960).

All of these methods (except possibly Kohn's) have been used by other workers to prepare extracts which were active in transformation. Details of the methods have already been presented. The preparations varied in purity, but samples containing approximately 1-5 µg./ml. DNA (estimated by Burton's method) were used in attempted transformation experiments.

Penicillin spheroplasts of the recipient strain A3(0)*his*$^-$ were prepared by the technique of Chargaff *et al.* (1957), in the presence of 1% (w/v) human serum albumin. Five 10 ml. portions of the spheroplast culture were measured out, and a different preparation of nucleic acid was added to each portion (see above for the five methods used for extraction). A further 10 ml. portion of the spheroplast culture was used as a control. All of the cultures were incubated at 37° for 2 hr., and then centrifuged in the cold. The cells were washed with cold 20% (w/v) sucrose, and resuspended in 20% (w/v) sucrose broth. Following overnight incubation at 37°, examination by phase contrast microscopy revealed that the cultures had reverted to rod-shaped forms. After centrifugation, the cells were washed and resuspended in 0.85% (w/v) NaCl. Aliquots were then plated out on minimal A, and the plates were incubated at 37° for 5 days.

Result

No indication of transformation to prototrophy was obtained from any of the cultures which had been converted to penicillin spheroplasts and then treated with nucleic acid extracted by five different methods from the prototroph.

Attempted transformation of streptomycin resistance

Although there a number of possible reasons for the negative results obtained above, one explanation could well be that in the strain used the mutation affecting histidine

biosynthesis could not be repaired by transformation. It was therefore decided to test another marker.

Nucleic acid was extracted from a streptomycin resistant mutant of A3(0) by the method of Gwinn and Thorne (1964), and aliquots were added to penicillin spheroplasts of W2 (*str*^{s}). The reversion procedure described in the last experiment was repeated, but in this case 0.2 ml. aliquots of the culture were spread on streptomycin nutrient agar.

Result

No colonies appeared on the streptomycin agar, and so transformation was not detected. At least 5×10^{9} cells were tested in each of two experiments.

Transformation of cells treated with agents active on cell wall structure

It has been mentioned above that there are a number of ways of preparing spheroplasts of gram negative organisms. Recipient cultures of A3(0)*his*$^{-}$ were treated by the lysozome/EDTA method, and by the freeze/thaw/lysozyme method. Nucleic acid was extracted from strains A3(0)*str*^{r} and A3(0) by the method of Gwinn and Thorne (1964). Aliquots containing 1-5 µg./ml. of DNA were added to separate portions of treated recipient cultures, and the mixtures were incubated at 37^{o} in 20% (w/v) sucrose broth until reversion to rod-shaped organisms appeared to be complete (about 4 hr.). Aliquots were then plated out on appropriate selective media. Cells

which had been incubated with an extract of the *str*^{r} donor were plated on streptomycin nutrient agar, while those which had been treated with an extract of the prototroph, A3(0), were plated on minimal A.

Result

No evidence of transformation of either marker was obtained.

EDTA treatment of recipient cells

It has been shown by Leive (1965b) that 2 x 10^{-4} M EDTA causes a non-specific increase in the permeability of *E. coli* without impairing viability, and she has suggested that this might facilitate the entry of transforming DNA. Although it was not known whether EDTA has a similar effect on *K. aerogenes* cells, the technique used by Leive was relatively simple to duplicate, and was therefore used to prepare recipient cells for transformation experiments.

The recipient culture, A3(0)*his*$^{-}$, was grown at 37^{o} in a minimal, Tris-Cl medium (Leive, 1965a) supplemented with histidine. The cells were harvested in early exponential phase, at a density of 2-4 x 10^{8}/ml., washed once with 0.12 M Tris-Cl, pH 8.0, and resuspended at a density of 2-5 x 10^{9}/ml. in the same buffer. Nucleic acid extracted from the prototroph A3(0) by Marmur's method was then added to a sample of the recipient suspension. EDTA was added to a final concentration of 2 x 10^{-4} M, and after 2 min. at 37^{o}, 10 volumes of

growth medium were added to terminate the treatment. In a second experiment, the nucleic acid was not added until 4 min. after the addition of 10 volumes of growth medium. Controls untreated with EDTA, with and without nucleic acid, were also included. All preparations were incubated at 37° for 2 and 4 hr., and samples were plated at these times on minimal A.

Result

The number of colonies on each plate corresponded to the reversion rate, as detected by the control experiments, and so no evidence of transformation was obtained. Detection of transformation under the above conditions would have been welcome, but there is some evidence to suggest that it might not be possible.

In recent papers, Hamilton-Miller (1965, 1966) has shown that EDTA has a profound effect on the permeability of K. aerogenes to benzylpenicillin, but fails to sensitize K. aerogenes to lysozyme. In E. coli, which can be sensitized to lysozyme by EDTA (Repaske, 1958), the action of EDTA is regarded as being due to chelation of bivalent metal ions combined with a stripping off of the lipoprotein and lipopolysaccharide layers which overlie the R (mucopeptide) layer (Hamilton-Miller, 1966). Hamilton-Miller proposes that if the stripping off process does not occur as much in K. aerogenes as in E. coli, then the limited amount which does occur might be sufficient to allow the small molecule of benzylpenicillin greatly facilitated access through the membrane complex, while

not allowing the much larger lysozyme molecule to reach the R layer. Accepting Hamilton-Miller's thesis, and assuming that transforming DNA would require to pass through the membrane complex in the same way as lysozyme, one can postulate that EDTA would only facilitate the passage of DNA fragments which have a molecular weight smaller than that of lysozyme. The molecular weight of egg-white lysozyme is approximately 1.5×10^4, whereas the mean molecular weight of a transforming preparation of DNA has been estimated to be 5×10^6 for the pneumococcal system, or 15×10^6 for the Haemophilus influenzae system (Hayes, 1964), and a minimal estimate of the size of an integrated piece of transforming DNA was 6×10^5 (Fox, 1962). It is possible therefore that fragments of DNA potentially capable of causing transformation might be too large to benefit from the damage caused by EDTA to K. aerogenes cell wall structures.

Apart from its effect on permeability properties in gram negative bacteria, there is evidence that EDTA can interfere with the transformation process in some established systems. Schlissel and Sword (1966) found that 10^{-5} M EDTA inhibited transformation of group H Streptococci, possibly by removal of necessary bivalent cations since Mn^{+2} effectively overcame the inhibition. On the other hand, Anagnostopolous and Spizizen (1961) found that 10^{-4} M EDTA can promote competence in Bacillus subtilis, and they suggested that this could be due to the removal of (presumably detrimental) cupric ions.

It therefore seems possible that transformation in

E. coli, and less probably in K. aerogenes, might be assisted by the disruptive action of EDTA on cell wall structure, but in the event that there are further requirements for competence analagous to those in Streptococci or B. subtilis, EDTA might also interfere at this stage.

Attempts to obtain "competent" Klebsiella cells

In species where transformation has been studied extensively, an important factor in the appearance of competence can be the stage in the growth cycle at which the recipient cells are treated with DNA. For example, in Bacillus subtilis systems, competence reaches a maximum at the end of the exponential phase of growth (Young and Spizizen, 1961), whereas Neisseria gonorrhoeae seems to be especially competent in the early exponential phase (Sparling, 1966). An experiment was therefore carried out in the hope of finding a period when Klebsiella cells are competent.

Nucleic acid was extracted from A3(0)str^r by the method of Gwinn and Thorne (1964). Aliquots of the extract were added to samples withdrawn from a yeast extract broth culture of A3(0)str^s at various stages of growth. The mixtures were incubated at 37° for 3 hr. to allow (a) possible incorporation of the str^r marker, and (b) phenotypic expression. Aliquots of each mixture were then plated on streptomycin nutrient agar, and the plates were incubated at 37° for 3 days.

Results

Not a single streptomycin resistant colony was recovered in this experiment, although samples of 2, 3, 4, 6, 8, 10, 12, 24 and 48 hr. cultures were treated with nucleic acid extracted from a *str*^{r} donor.

Attempted transformation of early log phase cultures of Klebsiella strains

In the absence of any indication that competence appears in a *Klebsiella* culture at any stage of growth, early log phase cultures were used in the next experiments. The aim was to test different recipients for the ability to be transformed. Nucleic acid extracted by the Gwinn and Thorne (1964) method from A3(0)*str*^{r} was used, and the recipients were 3 hr. YE/broth cultures of *Klebsiella* strains A3, A3(0), A3(0)*his*$^-$, 1.2, 1.9, 1.9(0), 2.12(0) and 5.6(0). Selection was for streptomycin resistance, but again no evidence of transformation was obtained.

To increase further the range of markers tested, nucleic acid extracted from A3(0) by the Gwinn and Thorne (1964) method was added to 3 hr. cultures of three different auxotrophs derived from A3 (*his*$^-$, *met*$^-$ and *pro*$^-$). After incubation at 37^o for 2, 4 and 16 hr., samples of the cultures were removed and the cells were washed in 0.85% (w/v) NaCl before plating on minimal A. Control cultures without nucleic acid were treated in the same way.

Result

No evidence of transformation was obtained in the above experiments, whether selection was for streptomycin resistant or prototrophic recombinants.

Attempted transformation of A3 auxotrophs on the surface of solid media

A screening method developed by Gwinn and Thorne (1964) for transformation of Bacillus licheniformis was adapted for use. The nucleic acid used in this experiment was extracted from the prototroph, A3(0), by Gwinn and Thorne's modification of Marmur's method. The auxotrophs A3(0)his$^-$ and A3pro$^-$ were tested separately for transformation to prototrophy.

Overnight broth cultures (10 ml.) of the auxotrophs were centrifuged, and the cells were resuspended in 10 ml. portions of 0.85% (w/v) saline. Samples (0.2 ml.) of the suspensions were spread on the surface of minimal A plates, with and without 0.1 ml. aliquots of nucleic acid from the prototroph. In a second experiment, cultures of the auxotrophs were converted to spheroplasts by the technique of Chargaff et al. (1957), washed in 20% (w/v) sucrose, and spread on 20% (w/v) sucrose-minimal A plates with and without nucleic acid. The plates were incubated at 37^o for 5 days, and then examined for transformants.

Result

If transformation had occurred under the above conditions,

more prototrophic colonies should have been found on the plates which had been treated with nucleic acid than on the control plates. This was not observed.

SECTION III

TRANSDUCTION

Among the Enterobacteriaceae, phage-mediated transduction has been reported to occur in strains of four genera: Escherichia (Morse, 1954; Lennox, 1955), Salmonella (Zinder and Lederberg, 1952), Shigella (Lennox, 1955), and Proteus (Coetzee and Sacks, 1960). Clarke (1961) has described possible transduction of a Klebsiella pneumoniae auxotroph to prototrophy, but this finding has not been confirmed, and a search of the literature has failed to reveal a single more definite report of transduction of a Klebsiella strain.

In his experiments, Clarke (1960, 1961) isolated 11 phages from pooled sewage, and used them in attempts to transduce 17 different Klebsiella auxotrophs. The only positive result which was obtained suggested that a cysteine-requiring auxotroph of strain 8172 could be transduced to prototrophy at low frequency, but there is a possibility that the rare prototrophs resulted from transformation by DNA present in the crude phage lysate. Treatment with deoxyribonuclease would confirm or eliminate this possibility, and before firm conclusions can be drawn about the system tests should be carried out with a variety of markers.

Despite the paucity of published reports, it seems likely that several workers have made unsuccessful attempts to establish transduction systems for Klebsiella strains. For example, tests have been carried out to determine whether the transducing Salmonella phage PLT-22 is capable of mediating transduction in systems involving Klebsiella strains, and temperate phages released by lysogenic Klebsiella strains have

been screened for transducing ability (C.H. Clarke; A.H. Stouthamer: unpublished results).

Klebsiella strains appear to be susceptible to infection by bacteriophages in the normal way, even although the host cell surface may often be occluded by a polysaccharide capsule. Studies of several phage/capsulate host systems have revealed that in many cases polysaccharide-decomposing enzymes are produced (Humphries, 1948; Park, 1956; Adams and Park, 1956; Sutherland and Wilkinson, 1965), and it has been suggested that these enzymes are capable of stripping the capsular polysaccharide from the surface of sensitive bacteria, thereby allowing phage particles to gain access to their recptor sites (Adams and Park, 1956). Thus it seems unlikely that Klebsiella strains will prove to be refractory to transduction simply because they are normally capsulate, but there is one possible difficulty which should be considered. The range of capsulate strains which a Klebsiella transducing phage can infect will almost certainly be restricted by the specificity of the phage-associated depolymerase, and, for example, it may prove impossible to carry out transductional analysis of mutant strains whose capsular polysaccharides have been altered sufficiently to render them insensitive to the depolymerase.

In the present study it is intended to look first for a transduction system which will allow genetic analysis of a widely used strain, Klebsiella aerogenes A3. This strain is not known to be lysogenic and there is no indication that it

is sensitive to a temperate phage. However, both of these possibilities can readily be tested, assuming that enough indicator strains and temperate phages are available. In the event that attempts to find a suitable temperate phage are unsuccessful, consideration may be given to virulent phages.

Transduction with virulent phage. It seems likely that the one possible case of transduction of a Klebsiella strain involved virulent phage, since the relevant phage was originally isolated from sewage and formed clear plaques on sensitive indicator bacteria (Clarke, 1960). Other virulent phages active on Klebsiella strains have been described by a number of workers, including Hadley (1925), Rakieten, Eggerth and Rakieten (1940), Humphries (1948), Park (1956), Keller and Olds (1958) and Milch and Deak (1964-65); virulent phages active on K. aerogenes strain A3 have been isolated by Clarke (1960) and Macgregor and Wilkinson (unpublished results). It has been found that most, if not all, of these phages produce plaques which are surrounded by translucent haloes and it is highly probable that the haloes result from the production of diffusible polysaccharide-depolymerases of the type mentioned above.

Transduction is theoretically possible with virulent phage because the genomes of transducing particles may, and often do, lack the determinants of certain phage functions (Starlinger, 1958; Hartman, 1963). Thus the transducing particle may enter the recipient cell and yet be incapable of

completing the lytic cycle. However, not all of the particles in a normal phage lysate will be defective, and so cells which have survived infection by a defective, transducing particle may still be killed by superinfection with complete, virulent particles. It is therefore necessary to protect possible transductants from superinfection, and this has been done successfully in a number of ways. For example, Zinder (1955) used lysogenic, immune recipients, Goldschmidt and Landman (1962) and Carere and Spada-Sermonti (1964) used ultraviolet-irradiated phage lysates, and Starlinger (1958) and Novick (1963) used low multiplicities of infection. However, the difficulties of working with virulent phage in transduction systems are such that it is preferable to use temperate phage if at all possible. This is particularly true in the initial stages, when reasonably rapid screening may only be possible if many different phages can be handled in a uniform manner.

Transduction with temperate phage. It has already been mentioned that phage-mediated transduction has been reported in strains of the genera Escherichia, Salmonella, Shigella and Proteus. Certain strains of each genus are susceptible to infection by phages which are competent in general transduction, and this means that virtually any small region of chromosome can be subjected to genetic analysis. In looking for a new system of this type, it should be possible to determine whether or not a phage is capable of transduction by

carrying out tests with only a few markers. However, the probability of any one marker being transduced is low (of the order of one transductant per 10^5 - 10^8 phage particles: Lennox, 1955; Hartman, 1963), and so the markers which are used must be reasonably stable, and must allow the application of selective techniques. Drug resistance and certain nutritional markers have been found to be particularly suitable. For example, if drug sensitive recipients are treated with phage which has been grown on a drug resistant strain, rare transductants can readily be selected by plating on drug-containing media.

It may be possible to detect a temperate phage which is capable of infecting cells of K. aerogenes strain A3, but if not it may be necessary to consider unrelated Klebsiella strains. A number of workers have claimed that several of their Klebsiella strains are lysogenic (Park, 1956; Ciuca et al., 1959; Eustatziou et al., 1960, 1962; Clarke, 1964; Milch and Deak, personal communication), but unfortunately some of these strains are unobtainable, and others may not be lysogenic at all. For example, several investigators (Stouthamer and Clarke, personal communication) have been unable to confirm lysogeny in certain of the strains described by Ciuca et al. (1959) and Eustatziou et al. (1960, 1962). This conflict is disturbing, and no complete explanation has yet emerged. One possibility is that the original reports were based on a test which would allow bacteriocinogenic strains to be mistaken for lysogenic strains. Both types of strain

release agents which produce zones of lysis on sensitive indicator bacteria and unless further tests are carried out it is not possible to decide whether the zones were caused by phages or bacteriocins. Thus Ciuca et al. (1959) and Eustatziou et al. (1960, 1962) may have thought that their strains were lysogenic zimply because culture supernatants contained agents which produced zones of lysis on sensitive bacteria, but this evidence is clearly not sufficient. In order to be certain that a strain is lysogenic, it is necessary to prove that it releases a phage; this can be done by demonstrating the formation of discrete plaques, or by showing that the strain releases a lytic agent which is capable of replicating in sensitive bacteria. When a number of the Klebsiella strains described by Ciuca et al. (1959) and Eustatziou et al. (1960, 1962) were tested in this way, none were found to be lysogenic (Stouthamer and Clarke, personal communication). Similarly, Clarke (1960) was unable to confirm that a particular type 2 Klebsiella strain was lysogenic, although Park (1956) had claimed that it was. However, the Kelbsiella strains described by Clarke (1964) and Milch and Deak (unpublished results) have been fully tested, and appear to be genuinely lysogenic. It is hoped that at least one of these strains will be found to release a phage which is capable of mediating transduction.

In looking for a new transduction system, phages may be grown to high titre, assayed by the standard techniques (Adams, 1959), and examined for their transducing abilities.

Alternatively, a method suggested by the classical studies of Zinder and Lederberg (1952) may be used. This method involves growing genetically marked bacterial strains together, and then examining for the formation of recombinants. The mechanism of the genetic exchange may then be clarified by further investigation, but if one of the parent strains is known to be lysogenic it may only be necessary to test lysates containing the relevant phage for transducing ability. When a new transducing phage is found, further tests should be carried out with a variety of markers so that it can be determined whether or not the phage is capable of general transduction. In certain circumstances, it may be possible to transduce two markers simultaneously, but it is likely that joint transductions will occur even less frequently than single transductions (Stocker, Zinder and Lederberg, 1953). Only those markers which are in close association on the chromosome can be transduced together, presumably because there is a limit on the size of the chromosomal fragment which can be incorporated into a phage particle. The amount of DNA normally contained by the average phage particle corresponds to approximately 1/100th of the genome of E. coli (Hayes, 1964), and it seems unlikely that a bacterial fragment of greater size than this would be transduced.

When strains of E. coli, Salmonella or Shigella are being used in joint transduction experiments, preliminary conjugation studies may provide an indication of whether two markers are closely linked and therefore likely to be co-transducible.

However, there is no conjugation system available for Klebsiella strains, and a search for linked transduction may have to be conducted empirically. Nevertheless, there are certain considerations which may help in studies with Klebsiella strains. Firstly, accurate and detailed chromosomal maps have been prepared for E. coli strain K12 (Taylor and Thoman, 1964), and since E. coli and Klebsiella strains have many phenotypic properties in common and may be reasonably closely related, it is possible that several regions of their chromosome maps are interchangeable with respect to gene order. Secondly, it has been established in many bacterial systems that functionally related genes have a marked tendency to be clustered together in the same chromosomal region (Demerec and Hartman, 1959; Hayes, 1964); application of this principle may provide valuable guidance in the choice of markers for co-transduction tests.

Several different methods of approach have been used in the many successful studies of linked transduction in bacteria. Of these, three appear to be particularly suitable for application to systems where there is little background information available at the outset. Firstly, a phage lysate propagated on a drug resistant, prototrophic donor strain may be used to transduce a drug sensitive, auxotrophic recipient strain. If streptomycin resistant prototrophs are recovered, evidence of linkage may be obtained. A modification of this procedure, involving transduction from streptomycin dependence to streptomycin independence, was used by Lennox (1955) to demonstrate

linkage of the loci for maltose utilisation and streptomycin independence. In the second approach, a series of doubly auxotrophic mutants may be isolated and tested for transduction to prototrophy with phage grown on a wild type (prototrophic) donor. Using this technique, Prozesky and Coetzee (1966) observed only two cases of co-transduction in studies with 111 double auxotrophs of Proteus mirabilis. The auxotrophs were all of the type arg$^-$ X$^-$, and it seems likely that similar attempts to determine linkage relationships between other markers would also require the initial isolation of large numbers of double auxotrophs. In the third approach, however, strains which have only one auxotrophic requirement can be used to study relationships between markers, and all possible combinations of the available markers can be tested directly. The method depends on the observation that linkage of markers on a single, transduced, bacterial, genetic fragment is reflected in a deficiency of wild type recombinants when two mutants are crossed (Demerec, Blomstrand and Demerec, 1955). In a proportion of the recombinant clones, the wild type allele is not recovered, since a mutant allele of the donor has jointly entered the recipient genome (Hartman, Loper and Serman, 1950; Hartman, 1963). A number of workers, including Clowes (1958), Yanofsky and Lennox (1959), Hartman et al. (1960) and Pearce and Loutit (1965) have obtained evidence of linkage in experiments based on this principle.

Methods

Phage lysates

Cultures which were thought to contain phage particles were centrifuged at low speed to deposit most of the bacteria. The supernatants were then rendered free of viable bacteria by membrane filtration or by shaking with chloroform.

Preparation of indicator plates

Two types of indicator plates were used:

(1) Nutrient agar plates were flooded with an overnight broth culture of the prospective indicator strain and allowed to dry ("dried lawns").

(2) An overnight broth culture of the prospective indicator strain was diluted 1/20 in fresh broth and incubated at 37° for a further 2 hr. An aliquot (0.1 ml.) of this culture was then used to seed a 2.5 ml. volume of molten soft agar held at 46°. After mixing by rotation the seeded medium was poured on to the surface of a nutrient agar plate and allowed to solidify by standing at room temperature for 30-60 min. ("soft seeded overlays").

Phage sensitivity tests

Indicator plates were prepared by one of the methods described above and thoroughly dried. Drops of the appropriate phage lysate were then placed on marked areas of the plates and allowed to dry. Zones of inhibition of growth or plaques were normally scored after overnight incubation at 37°.

Preparation of high titre phage lysates

Phage lysates were prepared by the soft agar layer method (Adams, 1959). A 10 ml. volume of molten soft agar held at 46° was seeded with 0.5 ml. of an overnight broth culture of the appropriate propagating strain and with 0.5 ml. of a suitable dilution of the initial phage preparation. After mixing by rotation the seeded medium was poured on to the surface of two nutrient agar plates (approximately 5 ml./plate) and allowed to solidify by standing at room temperature for 30-60 min. Semi-confluent lysis appeared after incubation at 37° for 6-8 hr. The overlays were then removed from the plates, homogenised in 5 ml. amounts of broth, and centrifuged at 3,000 g for 20 min. Supernatants obtained in this way were passed through membrane filters to remove residual bacteria and were regularly found to contain 10^{10} - 10^{11} plaque-forming particles per ml.

Titration of phages

(1) Surface counts. Serial tenfold dilutions of the phage lysates were prepared in sterile broth, and 0.02 ml. volumes of each dilution were spotted on to dried lawns of the indicator bacteria. The plates were incubated overnight at 37°, and then counts were made in drop areas which contained the largest numbers of plaques without confluence.

(2) Soft agar layer technique (Adams, 1959). The indicator bacteria and a measured amount of the phage dilution were mixed with 2.5 ml. of molten soft agar held at 46°. The mixture was then poured over the surface of a nutrient agar

plate, and plaques were scored after overnight incubation at 37°. Routinely, three plates were used per phage dilution.

Ultraviolet treatment of lysogenic bacteria

An overnight broth culture of the lysogenic strain was diluted 1/10 in fresh broth and incubated at 37° for a further 3 hr. The resulting culture was centrifuged, and the packed organisms were resuspended in saline. Aliquots (4.5 ml.) of the suspension were then pipetted into 10 cm. petri dishes and exposed to ultraviolet light for periods of 5, 10, 15, 20, 30 or 60 sec. at a distance of 25 cm. from the source. Irradiation was carried out in the dark, and the suspensions were agitated by hand throughout the exposure period. Under these conditions it was found that over 95% of the bacteria survived treatment for 5 sec., whereas less than 1% survived treatment for 60 sec.

After irradiation, the various suspensions were mixed with 0.5 ml. portions of X 10 broth and incubated at 37° in the dark. Samples were withdrawn at intervals during the incubation period and assayed for phage by the surface count method.

Isolation of lysogenic derivatives of sensitive bacteria

A dilute phage preparation (titre c. 1 x 10^6 p.f.p./ml.) was spot tested on dried lawns of sensitive bacteria as previously described. The plates were incubated at 37° for 16 hr., and it was found that turbid zones of lysis appeared in most of the drop areas. Some of these zones still contained viable bacteria, which were sampled with a sterile

loop and streaked out on fresh nutrient agar plates. Several of the resulting colonies were then picked and subcultured at least three times on solid media to free them of contaminating phage.

The final isolates obtained in this way were used to inoculate a series of broth tubes. Each culture was incubated at 37° for 16 hr., and after this time a sample was withdrawn, passed through a membrane filter, and spot tested on a dried lawn of the sensitive strain. A further sample of the culture was spread over the surface of a nutrient agar plate and tested for sensitivity to the original phage preparation. Isolates which were found to release phages active on the sensitive strain were tentatively assumed to be lysogenic if their cultures were resistant (immune) to the original phage. Further confirmation was usually obtained by restreaking the lysogenic derivatives on solid media and testing several single colonies for phage production.

Adsorption of phage

Overnight broth cultures of the test strains were diluted 1/10 in fresh broth and reincubated at 37° until they had grown to a density of approximately 2 x 10^9 cells/ml. (2-3 hr.). Dilutions of these cultures were made in saline, and viable counts were carried out by the method of Miles and Misra (1938). Aliquots (9.5 ml.) of the cultures were then mixed with 0.5 ml. aliquots of phage lysates which had previously been titrated by the soft agar layer method (Adams, 1959). The mixtures were incubated at 37° for 10 min. to allow adsorption to occur, and

then centrifuged in the cold at 8,000 g for 10 min. to remove bacteria plus adsorbed phage. Any bacteria remaining in the supernatants were removed by membrane filtration, and the filtrates so obtained were assayed for phage by the soft agar layer method. Measurement of adsorbed phage was made by difference.

Spot tests for transduction to prototrophy

The recipient auxotrophic bacterial strain was grown in broth overnight, and 0.1 ml. of the resulting culture was spread evenly over the surface of a minimal agar plate. A measured drop of a high titre phage lysate was then placed on the surface of the inoculated plate and allowed to dry without spreading. Drops of sterile broth were also placed on the plate, to ensure that any positive results were not due to residual nutrients in the phage lysates. Colonies appearing on the plates were scored after incubation at 37° for 48 hr. If transduction occurred, many more colonies were observed in the areas where phage had been applied than were observed in phage-free areas.

Transduction in broth

In a typical test, an aliquot of an overnight broth culture of the recipient strain was placed in a water bath at 37° to equilibrate, and an equal volume of a phage lysate from the donor strain was added. The mixture was held at 37° for 15 min. to allow phage adsorption to take place, and then 0.1 ml. samples were removed and spread over a series of singly-enriched minimal agar (SEM) plates. Two controls were

always carried out: (1) samples of the uninfected recipient culture were plated on SEM to provide a control for spontaneous mutants; (2) phage lysates were spotted on nutrient agar for sterility tests immediately before they were used.

Transduction to streptomycin resistance

Aliquots of overnight broth cultures of the recipient (*str*^{s}) bacteria were mixed with equal volumes of phage from the donor (*str*^{r}) strain. The mixtures so obtained were incubated at 37° for 15 min. to allow adsorption, and then treated in one of the following ways:

(1) Delayed selection technique (Coetzee and Sacks, 1960)

Samples (0.1 ml.) of the adsorption mixtures were spread to dryness on nutrient agar plates and incubated at 37° for 3-4 hr. The growth which appeared was then carefully overlaid with 5 ml. amounts of soft agar containing sufficient streptomycin to give a final concentration of c. 1,000 μg./ml. throughout the plates. Colonies were scored after incubation at 37° for 3-5 days.

(2) Replica plating technique (Watanabe and Watanabe, 1959)

Samples (0.1 ml.) of the adsorption mixtures were spread on nutrient agar plates, grown for 4-6 hr. at 37°, and then replicated with sterile velvet to nutrient agar plates containing 1,000 μg. of streptomycin per ml. of medium. Colonies were scored after incubation at 37° for 48 hr.

(3) Liquid culture technique (Watanabe and Watanabe, 1959)

The adsorption mixtures were diluted 1/10 in fresh broth and incubated at 37° for up to 16 hr. Samples (0.2 ml.) were

withdrawn at various times and spread over nutrient agar plates containing 1,000 μg. of streptomycin per ml. of medium. Colonies were scored after incubation at 37° for 48 hr.

In the above experiments, recipient cells were treated with phage grown through at least three cycles on a $\underline{str}^r$ donor strain, and then incubated in the absence of streptomycin for varying times before exposure to the drug. This procedure was adopted because it has been shown that transductants which have recently acquired the streptomycin resistance marker do not normally express their resistance immediately (Watanabe and Watanabe, 1959). Three controls were always performed: (1) phage lysates were spotted on nutrient agar plates for sterility tests immediately before they were used; (2) recipient cultures were mixed with sterile broth instead of with phage, and then treated in exactly the same way as the corresponding transduction mixtures; (3) recipient cultures were mixed with equal volumes of phage grown on a $\underline{str}^s$ host, and then treated in exactly the same way as the corresponding test mixtures. None of these control preparations were found to yield colonies on streptomycin agar plates.

Experiments and Results

Interactions between K. aerogenes strain A3 and temperate bacteriophages

For reasons which have already been mentioned, the organism which was selected for initial study was K. aerogenes strain A3. This strain was not known to be lysogenic, and there was no indication that it was sensitive to a temperate phage. However, most of the established transduction systems were discovered as a result of studies with temperate phages, and so it was decided to look for temperate phages which could interact with strain A3.

There are three main approaches to this problem. Firstly, strain A3 may itself be lysogenic; this possibility can be examined by testing A3 culture supernatants for lytic action on a number of other strains. Secondly, other strains may be lysogenic, releasing phages which can be propagated on strain A3; this possibility can be examined by testing appropriate culture supernatants for lytic action on strain A3. Thirdly, one can isolate phages from natural sources (e.g. sewage: Macgregor and Wilkinson, unpublished results) by enrichment with cultures of strain A3, and some of the phages so isolated may prove to be temperate.

Attempts to detect lysogeny in K. aerogenes strain A3

Lysogenic bacteria carry a stable hereditary determinant which renders them capable of producing phage, and they are

Table 3.1

Strain	Result	Strain	Result	Strain	Result	Strain	Result
1.2	-	2 Park	-	8808	-	W69	-
1.9	-	5.6	-	8821	-	W70	-
2.1	-	A1	-	8843	-	W71	-
2.2	-	A4	-	8852	-	W72	-
2.5	-	54.2	-	8866	-	G7	-
2.6	-	K66	-	5936	-	RVC 2907	-
2.7	-	57.10	-	W52	-	RVC 4925	-
2.12	-	418	-	W53	-	703	-
2.16	-	6869	-	W54	-	S121	-
2.22	-	8167	-	W55	-	S5	-
B7380	-	8172	-	W56	-	242	-

The strains which are underlined have been described as lysogenic by other workers.

Sensitivity of 44 strains to a culture filtrate of K. aerogenes A3

immune to lytic infection by this same phage. Thus in order to detect lysogeny it is usually necessary to find a sensitive, indicator bacterial strain on which the phage will form plaques. In this experiment, the aim was to test a collection of 44 strains for sensitivity to a phage which might be present in culture supernatants of strain A3.

Single colonies of strain A3 were inoculated into cotton-wool stoppered broth tubes and incubated for 16 hr. and 48 hr. at 37°. Aliquots of the cultures were centrifuged at low speed to sediment the cells, and the supernatants were passed through membrane filters (pore size 0.45 μ). The filtrates were then spot tested on nutrient agar plates which had been spread with overnight broth cultures of the 44 prospective indicator strains. After incubation at 37° for 8 hr. and 24 hr., the plates were examined for zones of inhibition of growth.

Results

The theoretical sensitivity of the above technique is such that it should have been possible to demonstrate the presence of as few as 10-20 p.f.p./ml. in the filtrate. However, strain A3 did not appear to release a phage active against any of the strains tested (see table 3.1).

Sensitivity of K. aerogenes strain A3 to phages released by other strains

In view of the above results, it was decided to examine the sensitivity of strain A3 to phages which might be present

in culture supernatants of various other bacterial strains. Lysogenic Klebsiella strains have been described by a number of workers, including Park (1956), Ciuca *et al.* (1959), Eustatziou *et al.* (1960, 1962), Clarke (1964) and Milch and Deak (personal communication). Several of these strains were obtained and included in this experiment.

The membrane filtration procedure used in the last experiment was satisfactory because only two culture supernatants had to be filtered. In this experiment it was intended to test 44 separate cultures for the presence of phage. Membrane filtration of all of these would have been lengthy and expensive, and so a simple technique described by Bertani (1951) was employed instead. Briefly, the principle of this technique is as follows: if the suspected lysogenic bacteria are sensitive to streptomycin, and the indicator bacteria are resistant, then growth of the lysogenic bacteria should be suppressed by streptomycin, but the ability of any free phage particles to form plaques should not be impaired. Spot tests can therefore be carried out directly on a streptomycin-containing medium.

The 44 test strains were grown in broth at 37^{o} for 48 hr. Measured drops of the resulting cultures were then placed on streptomycin nutrient agar plates which had previously been flooded with an overnight culture of strain A3*str*^{r} and allowed to dry. Zones of inhibition of growth or plaques were scored after incubation at 37^{o} for 24 hr.

Table 3.2

Strain	Result	Strain	Result	Strain	Result	Strain	Result
1.2	-	2 Park	-	8808	-	W69	-
1.9	-	5.6	-	8821	-	W70	-
2.1	-	A1	-	8843	-	W71	-
2.2	-	A4	-	8852	+	W72	-
2.5	-	54.2	-	8866	-	G7	-
2.6	-	K66	-	5936	-	RVC 2907	-
2.7	-	57.10	-	W52	-	RVC 4925	-
2.12	-	418	-	W53	-	703	-
2.16	-	6869	-	W54	-	S121	-
2.22	-	8167	-	W55	-	S5	-
B7380	-	8172	-	W56	-	242	-

Sensitivity of *K. aerogenes* A3*str*^{r} to lytic agents released by 44 strains

Results

The results are presented in table 3.2. It can be seen that 43/44 cultures did not appear to contain a lytic agent active on strain A3*str*^{r}. Several of the 43 cultures are known to be lysogenic, but the present results presumably mean that A3*str*^{r} is not sensitive to the phages which they release. The one strain which was found to interact with A3*str*^{r} was strain 8852 - a drop of an 8852 culture was found to cause confluent clearing of a small area on the A3*str*^{r} lawn. There are a number of agents which could cause clearing of this type, and so further investigation was necessary.

Interaction between *K. aerogenes* strain A3*str*^{r} and strain 8852

In the previous experiment, it was found that a drop of a broth culture of strain 8852 produced a zone of confluent clearing on a streptomycin agar lawn of strain A3*str*^{r}. Further spot testing showed that a membrane-filtered culture supernatant of strain 8852 produced similar zones of clearing on nutrient agar lawns of strain A3*str*^{r}. The zones were turbid, but did not appear to be composed of discrete plaques. However, high titre phage lysates often cause a similar type of confluent lysis when tested on a sensitive culture, and so it was decided to examine the effect of more dilute preparations of the 8852 filtrate.

Tenfold dilutions of the 8852 culture filtrate were prepared in sterile broth, and spot tested on dried lawns of strain A3*str*^{r}. It was found that the undiluted filtrate

caused confluent clearing as before, but none of the dilute preparations caused any clearing at all. Discrete plaques, indicative of the presence of phage, were not observed. This finding suggested that the agent which caused the zones of clearing was unable to replicate in A3*str*^{r} cells, and support for this view was obtained in a further experiment. Cores of agar were cut from zones of confluent clearing, and shaken in a small volume of broth. The eluate was passed through a membrane filter, and then added to a 3 hr. broth culture of A3*str*^{r}. After overnight incubation of this culture, it was found that the supernatant was devoid of lytic activity.

Results

A culture filtrate of strain 8852 contained an agent which caused clearing in a lawn of A3*str*^{r}, but the ability to cause clearing was readily diluted out, and the responsible agent could not be propagated in cultures of A3*str*^{r}. One possible explanation of this effect is that strain 8852 produces a bacteriocin-like substance. Bacteriocins generally produce zones of inhibition of growth when plated on sensitive bacteria, and the activity of a bacteriocin-containing preparation decreases on dilution. Alternatively, strain 8852 may be lysogenic, and the phage which it releases may be able to kill A3*str*^{r} cells without being able to multiply in them. Killing could result from damage to the host cell membrane by an excess of phage ("lysis from without": Adams, 1959), and in such a case one would expect the lytic activity of a preparation to diminish on dilution. A similar suggestion was made by Amati

(1962), when he found that phage P1 could kill cells of Pseudomonas aeruginosa and Serratia marcescens without multiplying in them.

No attempt was made to distinguish between the above possibilities, because it seemed unlikely that the interaction between strains 8852 and A3str^r would prove useful in establishing a transduction system for study of K. aerogenes strain A3. However, if strain 8852 is indeed lysogenic, and if one can find indicator bacteria which can support multiplication of the phage, then it may well be possible to carry out transduction studies with strain 8852 itself.

Interaction between K. aerogenes strain A3 and phage PW52

K. aerogenes strain W52 is lysogenic (see later), releasing a phage which has been called PW52. This phage was found to produce plaques on strain W70, and high titre lysates were readily obtained by the soft agar overlay method (Adams, 1959). It was then found that lysates containing more than c. 1 x 10^7 p.f.p./ml. caused turbid zones of clearing when spot tested on strain A3. However, discrete plaques were never observed when more dilute preparations were tested, and attempts to propagate PW52 on strain A3 were unsuccessful. It seemed likely, therefore, that PW52 caused "lysis from without" (Adams, 1959) when plated on strain A3.

Interaction between K. aerogenes strain A3 and the transducing phages λ and Plkc

High titre lysates of the phages λ and Plkc were spot tested on nutrient agar plates which had been spread with cultures of K. aerogenes strain A3. The plates were incubated at 30°, 37° or 42° for 16 hr. and 48 hr., and then examined for zones of lysis. It was found that neither of the phage lysates produced zones of lysis or inhibition of growth on lawns of strain A3.

Interaction between phage D5 and K. aerogenes strain A3

While working with K. aerogenes strain A3, Dr. I.W. Sutherland observed some unusual colonies on nutrient agar plates. These colonies were survivors of an A3 culture which had been treated with acriflavine, and were of interest because they had a number of peripheral semi-circular indentations or notches. We suspected that this phenomenon was caused by phage, and the colonies were therefore tested for lysogeny.

One of the notched colonies was purified by serial subculture on nutrient agar plates. Each subculture was made with a single colony which had clearly visible peripheral indentations, and after three successive transfers a stock culture (designated W43) was inoculated with a single colony of this type. When culture W43 was now streaked out on a nutrient agar plate, it was found that some of the resulting colonies had indentations and some did not.

Single colonies of the notched-edge type were used to inoculate broth cultures, and it was found by spot testing that the supernatants of such cultures contained a phage capable of forming plaques on lawns of strain A3. This phage was called D5. Single colonies of the entire-edged type were similarly tested, and in this case it was found that only 2/12 supernatants contained phage. Further spot tests revealed that the cultures which did not contain phage were in fact sensitive to phage D5.

Phage D5 was cultured by the usual methods, with strain A3 as host. After two successive single plaque isolations there appeared to be only one plaque-type, and so the phage culture was assumed to be pure. Discrete plaques were clear, but spot tests with a high titre (c. 1×10^9 p.f.p./ml.) lysate of the phage did not produce completely clear zones of lysis on lawns of strain A3. Surviving bacteria were picked from such a zone and subcultured three times to free them from contaminating phage, but no lysogenic clones were recovered.

From the results of the above experiments, it appeared that the relationship between strain W43 and phage D5 was almost certainly not lysogeny. However, it was decided to look briefly for evidence of genetic exchange mediated by phage D5. For this purpose, two different auxotrophic mutants (W43<u>arg</u>$^-$ and W43<u>pro</u>$^-$) were isolated following penicillin screening of an ultraviolet-treated culture of strain W43. These auxotrophs did not form colonies with notched edges, their broth cultures did not contain phage D5, and they were

both sensitive to this phage.

Aliquots (5 ml.) of overnight broth cultures of the auxotrophs were mixed in sterile tubes. The mixtures were either plated out directly on minimal agar, or diluted 1/10 in fresh broth and incubated for a further 16 hr. at 37° prior to plating. Each auxotroph was also diluted and plated separately, to provide controls for spontaneous reversion to prototrophy. The above procedure was then repeated, but this time 0.2 ml. of a phage D5 lysate (titre c. 5×10^6 p.f.p./ml.) was added to each auxotrophic culture before samples with withdrawn for mixing or for use as controls.

Results

It was found that mixed cultures of the auxotrophs W43*arg*$^-$ and W43*pro*$^-$ did not yield more prototrophs than were obtained from equivalent control cultures, whether phage D5 was present or not, and so there was no indication that phage D5 could act as a vector in the transfer of genetic information.

Isolation of phages from natural sources

In the previous experiments, it was not found possible to detect a temperate phage which could be propagated in cells of *K. aerogenes* strain A3. It was therefore decided to attempt to isolate a series of phages from natural sources by the enrichment culture technique. The host organism was normally strain A3, but occasionally a non-capsulate derivative of this strain was used instead.

A number of different samples of raw sewage were obtained

from the City Engineer, Potterrow, Edinburgh. An equal volume of broth was added to each sample to avoid inactivation of the bacteriophages on shaking (Adams, 1959). The mixture was then shaken with chloroform (1 vol. chloroform : 1 vol. mixture) to kill the bacteria present. After standing overnight in the cold, the mixture had formed two layers, and the top layer was used as the source of phages.

A 10 ml. broth culture of the potential host strain was incubated overnight and then diluted 1/10 in fresh broth and incubated for a further 1-2 hr. at 37°. To this culture was added 1 ml. of the sewage preparation and the mixture was incubated overnight at 37°. The culture was centrifuged briefly to remove most of the bacteria. Remaining bacteria were killed by shaking with chloroform, and the supernatant was then screened for phages active on the host strain. This was done by spreading aliquots of tenfold dilutions of the supernatant on nutrient agar plates which had been flooded with a culture of host bacteria and allowed to dry. After overnight incubation at 37°, the plates were examined for single plaques. Any well-isolated plaques were picked with a sterile wire into 1-2 hr. broth cultures of the appropriate host, and once again the cultures were incubated at 37° overnight. The chloroform sterilised supernatants of these cultures contained phages, which were purified by two further single plaque isolations. The phage preparations finally obtained appeared to be pure, and were used to obtain high titre lysates by the soft agar layer technique (Adams, 1959).

Table 3.3

Phage	Source	Plaque morphology on strain A3	Host strain used for isolation
D1	Cramond sewage	large, clear	A3
D2	Granton sewage	small, slightly turbid	A3
D3	Trinity sewage	medium, clear	A3(0)
D6	Cramond sewage	small, clear	A3(0)
D10	Trinity sewage	small, clear	A3
D11	Pig manure	large, irregular border	A3
D12	Pig manure	small, clear	A3
D13	Trinity sewage	small, clear	A3
D15	Pig manure	small, large halo	A3
*D16	Cramond sewage	small, clear	A3
*D17	Cramond sewage	large, clear	A3

* Obtained by membrane filtration method

Bacteriophages isolated by enrichment culture

Table 3.4

Phage	Source	Plaque morphology on strain A3	Host strain used for isolation
D4	Dr. C.H. Clarke	large, clear	54.2(0)
D5	strain W42	small, slightly turbid	A3
F31	Dr. I.W. Sutherland	clear	type 54
F32	Do.	clear	type 54
F33	Do.	clear	A3
F34	Do.	clear	A3

Bacteriophages active on K. aerogenes strain A3

The above procedure was repeated several times, using sewage from different parts of Edinburgh. Pig manure (obtained from the Veterinary Field Station, Easter Bush, Edinburgh) was used as an alternative starting material, and was found to be an excellent source of phages. In one experiment, removal of bacteria from supernatants was carried out by membrane filtration to preserve phages which might have been killed by the routine chloroform treatment.

Results

Nine phages were isolated using K. aerogenes strain A3 as host, and two using A3(0). The A3(0) phages both produced plaques when spot tested on plates which had been spread with cultures of strain A3, and so the capsule which normally surrounds a typical A3 cell is not a barrier to infection by these phages. The properties of the eleven phages are listed in table 3.3, and details of phages obtained from other workers are presented in table 3.4.

Attempted lysogenisation of K. aerogenes strain A3

Five of the phages previously described (D2, D3, D5, D15 and PW52) produced turbid zones of partial clearing when lysates containing approximately 1 x 10^7 p.f.p./ml. were spot tested on dried lawns of K. aerogenes strain A3. Samples were picked from such zones with a sterile loop and streaked out on nutrient agar plates. Several of the colonies which appeared were then purified by four successive single colony isolations, a procedure which should have been sufficient to

free the bacteria of contaminating phage. The colonies finally obtained were used to inoculate broth cultures, and after overnight incubation at 37° the supernatants of these cultures were screened for phage by the standard method.

Results

None of the culture supernatants were found to contain phages capable of forming plaques on strain A3, and so it was assumed that none of the clones had been lysogenised.

Attempted transduction of K. aerogenes strain A3 to streptomycin resistance

From the results of the previous experiments, it can be seen that virulent phages active on strain A3 were readily available, whereas all attempts to isolate temperate phages which could infect cells of this strain were unsuccessful. It was therefore decided to examine the possibility of using virulent phages in transduction of A3 derivatives.

Eight of the phages listed in tables 3.3 and 3.4 were propagated on strain A3(0)str^r by the soft agar overlay method (Adams, 1959). It was intended to infect the recipient cells at low multiplicities in this experiment, and so 1 ml. aliquots of the various phage lysates containing c. 1 x 10^9 p.f.p./ml. were mixed with 1 ml. aliquots of overnight broth cultures of strain A3 containing c. 2 x 10^9 cells/ml. (multiplicity c. 0.5). The mixtures, and controls of the bacterial cultures alone and the lysates alone, were incubated at 37° for 20 min. to allow phage adsorption. Triplicate 0.1 ml. samples were then

Table 3.5

Phage	Result
D1	-
D2	-
D3	-
D4	-
D5	-
D10	-
F31	-
F32	-

Attempted transduction of *K. aerogenes* A3 to streptomycin resistance

withdrawn from each tube and spread on nutrient agar plates. Further samples were diluted 1/10 in sterile broth before spreading on nutrient agar, in order to cut down the amount of reinfection by free phage. The plates were incubated at 37° for 4 hr. to allow possible *str*^{r} transductants to express their phenotype (Watanabe and Watanabe, 1959), and then soft nutrient agar containing sufficient streptomycin to give a final concentration of c. 1,000 µg./ml. throughout the medium was poured on as an overlay. Colonies were scored after incubation at 37° for 7 days.

Results

The results are presented in table 3.5. No evidence was obtained to suggest that any of the phages were capable of mediating transduction to streptomycin resistance.

Attempted transduction of auxotrophic derivatives of *K. aerogenes* strain A3

Five virulent phages (D1, D2, D3, D5 and D15) were propagated on the prototrophic strain A3. Lysates containing 1-2 x 10^{10} p.f.p./ml. were obtained, and these were used in attempts to transduce the auxotrophs A3*arg*$^-$, A3*pro*$^-$, and A3(0)*his*$^-$ to prototrophy.

The recipient auxotrophs were grown in broth overnight, and 0.1 ml. aliquots of the resulting cultures were spread to dryness on minimal agar plates. Drops of the various phage lysates were then placed on each plate and spread with a sterile loop over marked areas of the inoculated surface.

Dilutions (1/10 and 1/100) of the phage lysates were prepared, and drops of the dilutions were placed on a further series of inoculated plates. After the plates had been incubated at 37° for 15-30 min. to allow phage adsorption, drops of 0.5% (w/v) sodium citrate were spread over the phage treated areas. Control plates without added citrate were also set up. Prototrophic colonies were scored after incubation at 37° for 3 days.

Results

No evidence of transduction to prototrophy was obtained. A slight background growth of the auxotrophs was observed on most of the plates, and in some cases zones of lysis were faintly discernible in the areas which had been spread with phage. Adams and Luria (1958) found that sodium citrate prevented reinfection on plates by phage P1, but in the above experiment the addition of citrate did not appear to prevent lysis. It seemed likely, therefore, that any cells which had been transduced to prototrophy would have been lysed following reinfection by virulent phage particles.

Attempted transduction with ultraviolet irradiated phage

In 1962, Goldschmidt and Landman demonstrated transduction with high multiplicities of ultraviolet-inactivated bacteriophage. The phage which they used successfully was a virulent mutant of the transducing phage PLT-22, but when they attempted to use virulent phages which were unrelated to known transducing phages, the results were negative. Some time later, however, Carere and Spada-Sermonti (1964) were able to detect

transduction with ultraviolet-inactivated preparations of a virulent staphylococcal typing phage (No. 80). It was therefore decided to attempt to transduce an auxotrophic derivative of strain A3 to prototrophy, using ultraviolet treated lysates of virulent phages.

The virulent phages D1, D2, D3, D5 and D15 were propagated on the prototrophic strain K. aerogenes A3, and the lysates so obtained were diluted 1/10 in phage buffer (Glover, 1962) to decrease the concentration of ultraviolet-absorbing nucleic acids and other substances. The titre of each diluted lysate was then reduced from 1-2 x 10^9 p.f.p./ml. to less than 1 x 10^2 p.f.p./ml. by exposure to ultraviolet light. Spot tests for transduction were carried out by placing drops of the irradiated lysates on minimal agar plates which had been spread with an overnight broth culture of the recipient auxotroph, A3(0)his$^-$.

Results

The plates were examined after incubation at 37^o for 5 days, but no evidence of transduction to prototrophy was obtained. Goldschmidt and Landman (1962) were also unable to detect transduction in similar experiments with four Pasteurella pestis phages and an E. coli phage (T7). These results may indicate that none of the phages tested were capable of incorporating fragments of the bacterial chromosome into their own genomes. Another possible explanation is suggested by the finding that the host-killing property of certain phages is much more resistant to ultraviolet light

than is the viability of the phages (Luria and Delbruck, 1943; Adams, 1959). Thus the lysates used in the above experiment may well have contained particles which retained host-killing ability but had lost plaque-forming ability.

Attempted transduction of non-capsulate mutants of *K. aerogenes* strain A3

During this work, it was hoped that a method would be found for transferring genetic information between Klebsiella strains, and in particular it was hoped to transfer information relating to capsulation. Several independently isolated non-capsulate mutants of *K. aerogenes* A3 were available, and it was therefore decided to look for transduction of these mutants to capsulation.

Six of the virulent phages were propagated on the capsulate host strain A3. Aliquots of the phage lysates (1-3 x 10^9 p.f.u./aliquot) were then added separately to 5 ml., 8 hr. cultures of the non-capsulate mutants. After overnight incubation at 37°, samples from each tube were diluted 1/10 in broth containing A3(0) antiserum. The antiserum broth cultures were incubated at 30° for 24 hr., and it was noticed that a precipitate had formed at the base of each tube. Assuming that this precipitate was the result of agglutination of non-capsulate cells by the A3(0) antiserum, it was hoped that enrichment for capsulate transductants (or revertants) might have occurred towards the top of the tubes. Aliquots (0.5 ml.) were therefore withdrawn from the top of each tube,

Table 3.6

Non-capsulate mutants	Phage					
	D1	D2	D3	D5	D6	D10
KP 12	-	-	-	-	-	-
KP 17	-	-	-	-	-	-
KP 18	-	-	-	-	-	-
KP 30	-	-	-	-	-	-
KP 31	-	-	-	-	-	-
KP 32	-	-	-	-	-	-
KP 33	-	-	-	-	-	-

- = no mucoid transductants recovered

Attempted transduction of non-capsulate mutants of *K. aerogenes* strain A3

diluted suitably in saline, and spread over 1% glucose-EMB plates. The plates were examined after incubation at 30° for 2 and 4 days.

Results

Confluent growth was observed on plates which had been spread with dilutions of 10^{-4} or 10^{-3}, and isolated colonies with dilutions of 10^{-5} and 10^{-6}. No colonies having the mucoid colonial appearance typical of the donor strain (A3) were detected, although it was found in reconstruction experiments that one or two clones of A3 could readily be identified when plated with a large excess of non-capsulate mutants. A list of the strains and phages used in this experiment is presented in table 3.6.

Choice of strains and phages for further experiments

In the previous experiments, attention was focussed primarily on phages which were known to be active on K. aerogenes strain A3, but no evidence of transduction was obtained. It was therefore decided to extend the search for a transduction system to other Klebsiella phages, regardless of host specificity, and since lysogenic Klebsiella strains were known to exist it seemed likely that this time it would be possible to use temperate phages in the initial studies.

In preliminary tests, several strains which had been described as lysogenic were obtained from other workers and used to inoculate 10 ml. aliquots of nutrient broth. These cultures were incubated overnight at 37°, and centrifuged to

deposit the cells. The supernatants were passed through membrane filters, and then spot tested on soft agar overlays seeded with the 44 indicator strains employed in previous experiments. Zones of clearing or plaques were scored after incubation at 37° for 16 hr.

Results

None of the 44 strains in my collection showed signs of lysis when tested with filtrates of strains 1.2, 2.2, 2.5, 2.12, B7380 and 2-Park. However, certain of the indicator strains were found to be sensitive to lytic agents present in culture filtrates of strains W52-W56 (see table 3.7).

Tenfold dilutions of the five active culture filtrates were then prepared, and drops of each dilution were placed on soft agar overlays seeded with the appropriate sensitive bacteria. In most cases it was found that discrete plaques were observed when the more dilute preparations were tested, but in other cases the lytic activity gradually diminished on dilution. It was thought that the interactions which did not lead to plaque formation represented further examples of "lysis from without" (Adams, 1959), and so no detailed investigation was carried out. However, all five of the suspected lysogenic strains were found to release phages, and there appeared to be at least one propagating strain available for each phage (see table 3.7). It was therefore decided to test the various lysogenic strain/indicator strain systems more fully, in the hope that one of the phages would prove to be capable of mediating transduction.

Table 3.7

Culture filtrate	Indicator Strain					
	W68	W69	W70	W71	W72	1.2
W52	-	-	+	-	-	-
W53	-	+	+	-	-	-
W54	(+)	(+)	-	-	+	(+)
W55	-	-	-	+	-	-
W56	-	-	-	+	-	-

- = no zone of clearing

(+) = zone of clearing with undiluted culture filtrate, but no plaques with dilute preparation

\+ = zone of lysis and discrete plaques observed

Detection of lysogeny in Klebsiella strains

Preliminary screening for genetic transfer

The technique used in this experiment was suggested by the classical studies of Zinder and Lederberg (1952), and involved preparing genetically marked strains, growing them together, and examining for the formation of recombinants. Attempts were therefore made to obtain stable auxotrophic mutants of the lysogenic strains W52-56 and of the indicator strains W68-72. In the first experiment, five auxotrophs were isolated following penicillin treatment of ultraviolet irradiated cultures (Gorini and Kaufman, 1960), and in a further experiment one auxotroph (W70/A1) was isolated directly from a 2-aminopurine-treated culture of strain W70.

Overnight broth cultures of the various auxotrophs were centrifuged and washed twice in saline. Concentrated (x 10) saline suspensions of the cells were then prepared, and 0.1 ml. aliquots were spread singly and in pairs on the surface of minimal A plates. After incubation for 48 hr. at 37° the plates were examined for prototrophic colonies.

Results

The results are shown in table 3.8. In most cases mixtures and controls yielded roughly similar numbers of prototrophs, but three of the mixed cultures yielded many more prototrophs than the corresponding controls. The crosses which gave positive results involved derivatives of strains W52 and W70, and although no definite conclusions could be drawn at this stage it seemed likely that the phage released by strain W52 was capable of mediating transduction.

Table 3.8

	W70/A1	W71/S1	W52/S1
W52/S1	40	0	-
W52/S2	24	0	48
W53/S1	0	0	2
W55/S1	1	1	0

Each figure represents the number of prototrophs found on a single mixture plate, corrected for the reverse mutations of the individual strains.

Mixed cultivation of *Klebsiella* auxotrophs

Further experiments were therefore designed to test this possibility.

Induction of phage development in K. aerogenes strain W52

When cultures of certain lysogenic strains are exposed to suitable doses of ultraviolet light, the whole population lyses and releases phage particles (Lwoff, Siminovitch and Kjeldgaard, 1950). Lysogenic bacteria which behave in this way are said to be inducible, and the phenomenon is called induction. Many other effective inducing agents have since been found, including X-rays, nitrogen mustards, hydrogen peroxide and organic peroxides (Jacob and Wollman, 1959).

The first experiments which were carried out to test strain W52 for inducibility were unsuccessful, because treatment of exponential cultures of this strain with widely varying doses of ultraviolet light (see Methods section) did not lead to lysis, and increased production of phage was never observed. However, in the course of another experiment it was noticed that a culture of strain W52 had lysed completely several hours after treatment with 25 µg./ml. of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). A non-lysogenic strain which had been treated in exactly the same way did not lyse, and so it was suspected that lysis of strain W52 had been caused by induction of phage development. The experiment was therefore repeated, but this time the W52 culture was assayed for phage before the addition of NTG and again after lysis had occurred. It was found that the phage titre increased from 2×10^5

p.f.p./ml. to approximately 5 x 10^9 p.f.p./ml. A control preparation of strain W52 which had not been incubated with NTG, but which had been treated in exactly the same way as the test preparation in all other respects, did not show an increase in phage titre.

Allan and McCalla (1966) have recently reported similar results with a lysogenic strain of E. coli, and so it seems likely that NTG is in fact an effective inducing agent. Nevertheless, phage lysates obtained by NTG treatment of W52 cultures were not considered suitable for use in the present study, because it was felt that the potent mutagenic activity of even trace amounts of NTG (Adelberg et al., 1965) might seriously distort the results of transduction experiments.

Isolation and propagation of the phage released by K. aerogenes strain W52

In order to carry out accurate transduction experiments, it is necessary to obtain high titre preparations of the relevant phage. From the results obtained above it seemed unlikely that the induction technique would prove useful in this respect, and so it was decided to attempt to propagate the phage by lytic passage through sensitive cells.

An overnight broth culture of strain W52 was centrifuged briefly at low speed to remove most of the bacteria, and the supernatant was passed through a 0.45 μ membrane filter. Tenfold dilutions of the filtrate were then prepared, and a 0.1 ml. sample from each dilution was spread over a nutrient

agar plate which had been flooded with a culture of strain W70. After incubation at 37° for 16 hr., single well-isolated plaques were clearly visible on certain of the plates. Only one plaque-type was observed, but this did not necessarily mean that only one type of phage was present in the original culture supernatant. Any other phage released by strain W52 might well have been unable to form plaques on strain W70, and would not have been detected. Thus in order to be reasonably certain that a preparation contained only the plaque-forming phage it was necessary to carry out a purification procedure.

Material from a single typical plaque was picked with a sterile needle, and inoculated into a 6 hr. broth culture of strain W70. After incubation for 16 hr. at 37°, the bacteria were removed from the culture by centrifugation and membrane filtration as before. Aliquots of tenfold dilutions of the filtrate were then spread on plates which had previously been seeded with a culture of W70, and once again single well-isolated plaques were observed. It seemed likely that the plaques were already free from contamination at this stage, but the whole isolation procedure was repeated twice more before it was assumed that the phage culture was pure.

The final culture filtrate obtained in the above experiment was used as the starting material for all further work. The phage which had been isolated was called PW52, and high titre preparations (containing 10^{10} - 10^{11} p.f.p./ml.) could be readily obtained by the soft agar layer technique (Adams, 1959) with W70 or a mutant of W70 as the host organism.

Table 3.9

Recipient	Donor strain	
	W70	W70/A1
W52/S1	+	+
W52/S2	+	+
W70/A1	-	-
W70/A1(PW52)	+	-

Spot tests for transduction to prototrophy

Assay of phage PW52 presented no particular difficulties, because the plaques which it formed were normally large and sufficiently clear to be distinguished with the naked eye.

Attempted transduction with phage PW52

The results of a previous experiment suggested that genetic transfer occurred when auxotrophs of the Klebsiella strains W52 and W70 were incubated in pairs on minimal agar. The mechanism of the genetic transfer was not clarified at the time, but since one of the parent strains was known to be lysogenic it seemed likely that phage-mediated transduction was involved. It was therefore decided to test the phage released by strain W52 for transducing ability.

High titre lysates were prepared by propagating phage PW52 in cells of the prototrophic strain W70, and in cells of W70/A1, an auxotrophic derivative of W70. The lysates so obtained were then used to carry out spot tests for transduction to prototrophy, with strains W52/S1, W52/S2, W70/A1 and W70/A1 (PW52) as the auxotrophic recipients. Prototrophic colonies were scored after incubation at 37^{o} for 48 hr.

Results

The results are presented in table 3.9. A positive result in this table indicates that many more prototrophic colonies were observed in the areas to which phage had been added than were observed in phage free areas. In this way evidence was obtained to suggest that strains W52/S1, W52/S2 and W70/A1 (PW52) could be transduced to prototrophy by phage

grown on W70, whereas strain W70/A1 could not. When the W70/A1 test plates were examined, it was found that zones of lysis were just visible in the phage-treated areas, and so it seemed likely that any transductants would have been lysed. On the other hand, strain W70/A1 (PW52) was a derivative of W70/A1 which had been lysogenised with PW52 and was therefore immune to the phage; this strain did not show signs of lysis and appeared to be readily transducible. The specificity of the genetic exchange was also established in this experiment, because it was found that phage grown on strain W70/A1 gave positive results when spot tested on heterologous recipients, but negative results when tested on the homologous recipient W70/A1 (PW52).

Treatment with deoxyribonuclease

Phage PW52 was propagated in cells of the prototrophic strain W70 as previously described. An aliquot (1 ml.) of the resulting lysate was mixed with 0.2 ml. of broth containing sufficient DNase to give a final concentration of 40 µg./ml., and incubated at 37° for 1 hr. A further 1 ml. aliquot was mixed with 0.2 ml. of broth and incubated at 37° for 1 hr. to provide an untreated control. Transduction experiments were then carried out by the broth adsorption method, the recipient being W52*ade*-3, an adenine-requiring mutant of strain W52.

Results

The results are presented in table 3.10. It can be seen that prior incubation with DNase did not cause a reduction in

Table 3.10

Recipient	No. of prototrophs/ml.		
	Control	+ Phage	+ Phage + DNase
W52/*ade*-3	11	136	193

Effect of prior incubation with deoxyribonuclease on a transducing lysate

Table 3.11

	Titre of phage (p.f.p./ml.)	No. of transductants/ml.
Initial preparation	8×10^8	0
Supernatant	3×10^7	0
Sediment fraction	3×10^9	360

Effect of high-speed centrifugation on a dilute phage lysate

the number of prototrophs which were detected. There was in fact a slight increase, and when Lennox (1955) observed a similar effect in experiments with phage P1 he suggested that it was due to "unclumping" of the phage or to cleaning of some phage particles so as to improve adsorption to the bacteria. However, the main conclusion which was drawn from this experiment was that the genetically active fraction in the phage lysate was not sensitive to DNase and was therefore unlikely to be naked DNA.

Centrifugation of a lysate containing phage PW52

Phage PW52 was propagated on the prototrophic strain W70 as previously described, and the resulting lysate (titre c. 8×10^8 p.f.p./ml.) was centrifuged at 65,000 g for 60 min. After this time, the supernatant fluid was carefully removed and the pellet was resuspended in a small volume of broth. The supernatant and sediment fractions were then passed through membrane filters to remove contaminating bacteria, and assayed for phage by the soft agar layer technique (Adams, 1959). To test for transducing activity, 0.02 ml. drops of each preparation were placed on minimal agar plates which had previously been seeded with a culture of the auxotroph W52ade-3.

Results

The results are presented in table 3.11. It can be seen that no transducing activity could be detected in the original lysate. However, when the lysate was centrifuged, the sediment fraction was found to acquire transducing ability as well

as a high plaque-forming titre. This result suggested that there was an association between transducing ability and plaque-forming particles, and it therefore seemed likely that it would be possible to obtain very high titre transducing preparations simply by centrifuging dilute lysates at 65,000 g for 60 min.

Frequency of transduction

A number of experiments were carried out to allow the frequencies of transduction of different markers to be estimated. Aliquots of overnight broth cultures of the auxotrophs W52ade-1, W52ade-3 and W52lys were mixed with equal volumes of a lysate of phage PW52 grown on the prototrophic strain W70. The mixtures were incubated at 37° for 15 min. to allow adsorption of the phage, and then 0.1 ml. samples were spread over a series of singly-enriched minimal agar plates to select prototrophic transductants. Mixtures containing sterile broth instead of the phage lysate were also incubated and plated to provide controls for spontaneous mutants. The numbers of viable bacteria in the original recipient cultures were measured by the method of Miles and Misra (1938), and the PW52 lysate was assayed for phage by the soft agar overlay technique (Adams, 1959).

Results

In preliminary experiments with phage PW52 and strains W52ade-1, W52ade-3 and W52lys, it was found that over 90% of the plaque-forming particles were adsorbed within 10 min. (see

Table 3.12

Recipient strain	No. of bacteria/ml.	Plaque-forming titre of phage lysate	% of phage adsorbed	No. of transductants per ml.	Transduction frequency
W52*ade*-1	2×10^9	2×10^9	c. 90	880	5×10^{-7}
W52*ade*-3	1×10^9	8×10^9	c. 90	369	5×10^{-8}
W52*lys*	1×10^9	2×10^9	c. 90	1480	8×10^{-7}

Estimation of transduction frequency

Methods section). The transduction frequencies were therefore calculated on the basis of 90% adsorption, and the results are shown in table 3.12. These results are of the same order of magnitude as the transduction frequencies reported by Lennox (1955) for phage P1.

Attempted transduction of non-mucoid mutants of strains W52 and W70

K. aerogenes strains W52 and W70 were found to form large, convex, glistening, mucoid colonies on nutrient agar or EMB-glucose. Examination in India ink films (Duguid, 1951) revealed that both strains were capsulate. A series of non-mucoid mutants of these strains were then isolated as previously described, and it was found that all such derivatives were non-capsulate.

In experiments designed to detect transduction to capsulation, each non-mucoid strain was grown in broth at 37° overnight, and 0.2 ml. of the resulting culture was spread evenly over the surface of an EMB-glucose plate. Measured drops (0.02 ml.) of a high titre preparation of phage PW52 grown on the mucoid strain W70 were then placed on the surface of the plate and allowed to dry without spreading. The plate was incubated at 37° for 48 hr. and examined for mucoid colonies.

Results

The results are presented in table 3.13. No mucoid colonies were observed in any of the phage treated areas, or indeed in the untreated control areas of the test plates.

Table 3.13

Strain	Result
W94	-
W95	-
W96	-
W100	-
W101	-
W103	-

(a)

Strain	Result
W93	-
W97	-
W98	-
W99	-
W102	-
W104	-

(b)

The strains listed in table (a) are non-mucoid mutants of strain W52, and those in table (b) are non-mucoid mutants of strain W70.

- = no mucoid transductants observed.

Attempted transduction of non-mucoid mutants of strains W52 and W70

In reconstruction experiments, serial tenfold dilutions of a culture of the mucoid parent strain were spotted on plates which had been seeded with a culture of the non-mucoid strain. The plates were incubated at 37^{o} for 48 hr., and after this time it was found that mucoid colonies could readily be distinguished against the background of non-mucoid growth. This result suggested that growth of mucoid transductants would not have been inhibited by the surrounding non-mucoid bacteria on the test plates.

Transduction to streptomycin resistance

Phage PW52 was propagated through three successive cycles in cells of strain W70*str*^{r}, and the resulting high titre lysate was used in attempts to transduce several other strains to streptomycin resistance. The recipient strains were W52, W70 and a series of non-mucoid derivatives of these strains. The three methods used for selection of streptomycin resistant transductants have already been described.

Results

The results are presented in table 3.14. It can be seen that streptomycin resistant transductants were detected in all three experiments involving the mucoid strain W52. The negative results obtained with strain W70 can be explained, because it has already been shown that cultures of this strain are partially lysed when treated with phage PW52, and it might therefore be expected that any W70 cells which had acquired the *str*^{r} marker in the above experiment would have been lysed

Table 3.14

(a)

Strain	Delayed selection (overlay) technique	Replica plating technique	Liquid culture technique
W52	+	+	+
W94	N	N	-
W95	-	-	-
W96	-	-	-
W100	N	-	-
W101	N	-	-
W103	N	-	-

(b)

Strain	Delayed selection (overlay) technique	Replica plating technique	Liquid culture technique
W70	-	-	-
W93	-	-	-
W97	-	-	N
W98	-	-	N
W99	-	N	N
W102	-	N	N
W104	-	N	N

+ = streptomycin resistant transductants recovered

- = no streptomycin resistant transductants recovered

N = not tested

Transduction to streptomycin resistance

following infection by free phage. The same explanation may apply to the non-mucoid derivatives of W70. However, the failure to detect streptomycin resistant transductants in experiments with the non-mucoid derivatives of strain W52 cannot be attributed to reinfection by free phage, because these derivatives are lysogenic and therefore immune to lytic infection by PW52.

Attempted transduction of a non-mucoid auxotroph of strain W52

In this experiment, attempts were made to transduce strains W52ade-3 and W108 (a non-mucoid mutant of W52ade-3) to prototrophy. The donor lysate was obtained by propagating phage PW52 in cells of strain W70, and the tests were carried out by the spot transduction method on minimal agar plates.

Results

It was found that the mucoid strain W52ade-3 could be readily transduced to prototrophy, whereas the non-mucoid derivative could not. Further tests were then carried out by the more sensitive broth transduction method, but the results were again negative when the non-mucoid derivative was treated with phage grown on strain W70.

A possible explanation for the behaviour of non-mucoid mutants of strains W52 and W70 in transduction experiments was suggested by the finding that a PW52 lysate which had a titre of 5×10^{10} p.f.p./ml. when plated on W70 appeared to have a titre of only 1×10^{6} p.f.p./ml. when plated on W93 (a non-mucoid derivative of W70). The reason for this marked

difference in efficiency of plating was not established, but it seemed likely that if only a proportion of the phage particles in a PW52 lysate were capable of forming plaques on a non-mucoid strain, then the transducing titre of the lysate with respect to non-mucoid recipients might well be correspondingly reduced.

Transduction of streptomycin resistance and maltose utilisation markers

In E. coli K12, conjugation studies have shown that a particular maltose utilisation marker (mal-1) is closely linked to the streptomycin resistance (str^r) marker (Cavalli-Sforza, Lederberg and Lederberg, 1953), and more recently it has been shown that these markers are in fact co-transducible (Lennox, 1955). It was therefore decided to look for a similar linkage relationship in K. aerogenes strain W52.

Five mal^- mutants of strain W52 were isolated by plating NTG-treated cultures directly on EMB-maltose, and when the mutants had been purified by serial subculture it was found that three of them were reasonably stable. In preliminary transduction experiments, drops of a high titre lysate of phage PW52 grown on strain W70str^r(mal^+) were placed on EMB-maltose plates which had been spread with cultures of the three stable mal^- mutants. After incubation at 37° for 16 hr., mal^+ transductants could be readily distinguished in the areas which had been treated with phage. Over 1,000 of the mal^+ transductants were then tested by replica plating to

EMB-maltose-streptomycin agar, and it was found that none of them were capable of growing. The few colonies which did appear on the streptomycin agar were unable to utilise maltose, and presumably represented *mal*$^-$ clones which had been transduced (singly) to streptomycin resistance.

In a second experiment, the various *mal*$^-$ mutants were again treated with phage grown on W70*str*^{r}, but this time the broth transduction method was used. Selection for *mal*$^+$ transductants was accomplished by plating on a medium which contained maltose as the sole carbon and energy source (i.e. M9-minimal-maltose: Adams, 1959), and in this way approximately 500 *mal*$^+$ clones were obtained. However, none of these clones were found to be streptomycin resistant when tested by replica plating to EMB-maltose-streptomycin agar.

Results

The results of the above experiments provided no evidence to suggest that the *str*^{r} and *mal* markers are linked in *K. aerogenes* strain W52. There are several possible explanations for this finding. Firstly, even if the *str*^{r} and *mal* markers are closely linked, any fragment of genetic material which contains both markers may be too large to be incorporated into a single PW52 particle. Secondly, it may be necessary to screen extremely large numbers of transductants in order to find one which has received both markers, because the frequency of single transduction of the *str*^{r} marker is extremely low (of the order of 3×10^{-9} per phage particle adsorbed) and the frequency of joint transduction is presumably even lower.

Finally, it is possible that the markers tested in the above experiment are widely separated on the W52 chromosome, in which case they would resemble the mal-5 and str^r markers of E. coli rather than the mal-1 and str^r markers (see Hayes, 1964).

Transduction of nutritional and streptomycin resistance markers

In order to isolate a series of auxotrophic mutants, cultures of strain W52 were treated with NTG as previously described. The method of Adelberg et al. (1965) proved extremely satisfactory for this work, and stable auxotrophs could be obtained very readily. Unfortunately, however, there was a high probability that every NTG-treated cell would have been mutated at more than one site (Adelberg et al., 1965), and it was therefore necessary to check that the lesions responsible for auxotrophy were susceptible to repair by transducing phage. This could be done directly for derivatives of strain W52, but derivatives of W70 had to be lysogenised with phage PW52 before transduction tests could be carried out. It was found that about half of the isolates gave prototrophic transductants when spot tested on minimal agar plates (see table 3.15), and only isolates of this type were considered suitable for use in later experiments.

Aliquots of overnight broth cultures of the various transducible auxotrophs were mixed with equal volumes of a preparation of phage PW52 grown on strain W70str^r. The

Table 3.15

Strain	Result	Strain	Result	Strain	Result	Strain	Result
L1	+	N1	-	N17	-	N33	+
L2	-	N2	-	N18	-	N34	-
L3	-	N3	+	N19	-	N35	-
L4	-	N4	-	N20	-	N36	+
L5	+	N5	+	N21	+	N37	+
L6	-	N6	+	N22	+	N38	-
L7	-	N7	+	N23	+	N39	-
L8	+	N8	-	N24	-	N40	-
L9	+	N9	+	N25	-	N41	-
L10	-	N10	+	N26	-	N42	-
L11	-	N11	+	N27	-	N43	+
L12	+	N12	-	N28	-	N44	-
L13	+	N13	-	N29	-	N45	+
L14	+	N14	+	N30	+	N46	+
L15	+	N15	+	N31	+	N47	-
L16	+	N16	-	N32	+	N48	+
L17	+						
L18	-						

L/1 - L/18 are auxotrophs of strain W70

N/1 - N/48 are auxotrophs of strain W52

Auxotrophic derivatives of K. aerogenes strains W52 and W70 - spot tests for transduction to prototrophy

mixtures were incubated at 37° for 15 min. to allow adsorption, and then 0.1 ml. samples were spread over a series of SEM plates. Controls were plated as previously described. The plates were incubated at 37° for 48 hr., and after this time any prototrophic colonies which had appeared were counted. It was found that most of the tests yielded over 150 prototrophic transductants.

The colonies which appeared on the test plates were then replicated with sterile velvet to streptomycin-minimal agar. Most of the streptomycin-minimal plates were barren, even after incubation at 37° for 7 days, but in two cases a few colonies were detected within 48 hr. The two positive tests were then repeated on a larger scale, and control experiments (involving treatment of the recipients with phage grown on the streptomycin sensitive strain W70) were carried out to test the specificity of the transducing lysate.

Results

The results are presented in tables 3.16 and 3.17. In the preliminary screening experiment, it was found that treatment of the ade-1 and ade-3 derivatives of strain W52 with phage grown on W70str^r led to the detection of colonies on streptomycin-minimal medium. Several of these colonies were tested by restreaking on the same medium, and they appeared to be streptomycin resistant prototrophs. It seemed possible therefore that the ade$^+$ and str^r markers from the donor strain had jointly entered a proportion of the recipient cells, but this explanation was found to be inadequate when it was shown

Table 3.16

Strain	Result	Strain	Result	Strain	Result	Strain	Result
L1	-	L16	-	N11	-	N32	-
L5	-	L17	-	N14	-	N33	-
L8	-	N3	-	N15	-	N36	-
L9	-	N5	-	N21	-	N37	-
L12	-	N6	-	N22	+	N43	-
L13	-	N7	+	N23	-	N45	-
L14	-	N9	-	N30	-	N46	-
L15	-	N10	-	N31	-	N48	-

Transduction mixtures were plated on SEM, and colonies appearing on this medium were replicated to streptomycin-minimal agar. A positive result in the above table indicates that colonies were detected on the streptomycin minimal plates.

Screening for joint transduction of streptomycin resistance and nutritional markers

Table 3.17

Recipient strain	Donor strain			
	W70		W70*str*^{r}	
	Min. A	Min. + str.	Min. A	Min. + str.
W52/N7(*ade*-1)	450	2	520	3
W52/N22(*ade*-3)	376	6	559	2

Treatment of *K. aerogenes* auxotrophs with phage grown on streptomycin resistant and streptomycin sensitive hosts

that treatment of the *ade*$^-$ mutants with phage grown on a streptomycin sensitive host (W70) also led to the detection of colonies on streptomycin-minimal medium (see table 3.17). Furthermore, a W52*ade*-3 culture which had not been treated with phage at all was found to yield several hundred small (c. 1 mm.) colonies when approximately 2×10^9 cells were plated on streptomycin-minimal agar. The small colonies did not become visible until the plates had been incubated at 37° for 3-5 days, but serial subcultures to the same medium became visible within 24 hr. (at 37°). Thus it is not necessary to postulate a specific joint transduction of the *ade* and *str*^{r} markers in order to explain the recovery of colonies on streptomycin-minimal agar. Finally, it should be mentioned that the sensitivity of the screening technique was such that any marker which was linked to the *str*^{r} marker should have given positive results if the frequency of joint transduction was greater than 1-2%, but if the frequency was less than this the results would probably have been negative.

Linkage relationships between nutritional markers

In previous experiments, attempts were made to detect co-transduction of the *str*^{r} marker with various other genetic markers, but no positive results were obtained. It was therefore decided to carry out a series of crosses between auxotrophic mutants, in the hope that some linkage relationships would become apparent. Several auxotrophs of strains W52 and W70 had already been isolated, and 22 of these were

chosen for use in this experiment.

High titre lysates were prepared by growing phage PW52 on each of the ten auxotrophic derivatives of strain W70, and on the wild-type W70. Most of the lysates were found to contain 2-5 x 10^{10} p.f.p./ml. when assayed on W70, but some preparations had to be concentrated by centrifugation at 65,000 g for 1 hr. in order to obtain a sufficiently high titre. Transduction experiments were then carried out by the broth adsorption technique, the recipient strains being 12 auxotrophs of W52 and 10 lysogenised derivatives of the W70 auxotrophs.

Overnight broth cultures of the various recipient strains were prepared and were consistently found to contain 1-2 x 10^9 cells/ml. In a typical test, a recipient culture was placed in a waterbath at 37° to equilibrate, and an equal volume of a phage lysate containing 2 x 10^{10} p.f.p./ml. was added (multiplicity 10-20). The mixture was incubated at 37° for 15 min. to allow phage adsorption, and then 0.2 ml. samples were removed and plated on singly-enriched minimal agar. At least four plates were used for each test, and control experiments were carried out as previously described. Colonies were scored after incubation at 37° for 48 hr.

Each recipient strain was transduced with phage grown on each of the 10 suitable donor auxotrophs, and with phage grown on the wild-type W70. There was no satisfactory method available for obtaining high titre lysates from W52 auxotrophs, and so such strains were not used as donors.

Table 3.18

Recipient	Donor strains:										
	ade-5	ilva-2	ilva-3	thr-2	thi-2	thi-3	leu-1	leu-2	pro-1	pro-2	wild-type
ade-1	142	22	c	126	470	100	156	264	270	504	675
ade-2	12	6	c	40	62	80	52	46	78	62	116
ade-3	134	80	286	246	232	212	286	190	232	384	625
ade-4	0	10	c	68	0	4	46	14	38	30	71
ade-5	0	0	4	18	0	5	28	8	15	10	8
ilva-1	30	4	c	20	58	0	50	22	44	42	52
ilva-2	85	0	63	5	23	0	0	4	25	5	13
ilva-3	55	8	0	85	35	23	5	67	100	123	33
thr-1	23	4	25	0	49	8	7	0	37	1	50
thr-2	5	0	8	0	8	0	5	0	0	5	4
thi-1	0	2	c	10	0	0	10	8	26	44	88
thi-2	0	0	0	0	0	0	0	0	0	0	0
thi-3	0	18	0	1	0	0	0	28	0	1	33
leu-1	150	0	c	80	65	0	0	0	120	108	330
leu-2	246	16	69	161	206	14	6	4	252	131	246
pro-1	54	34	c	91	89	36	159	94	0	16	61
pro-2	28	31	43	21	116	28	56	68	18	0	173
cys.met	138	36	c	130	300	238	204	104	138	194	293
met	0	2	c	12	4	2	28	16	26	0	72
arg	362	30	c	174	496	48	420	234	484	546	498
lys	540	155	c	415	825	105	435	210	445	665	730
ura	380	145	266	350	274	195	330	353	492	290	360

c - phage preparation was contaminated

These figures are the total transductants on four plates, corrected for the reverse mutations of the recipient strains.

Transduction tests with Klebsiella auxotrophs

Results

The results are presented in table 3.18. The interpretation of these results depends upon the observation that linkage of markers on a single transduced genetic fragment may be reflected in the recovery of fewer prototrophs in a mutant x mutant cross than in a mutant x wild-type cross (Demerec et al., 1955; Hartman, 1963). In this experiment, it was found that certain of the recipient strains (e.g. thr-2, ade-2) yielded very few transductants in mutant x wild-type crosses, and so no attempt was made to assess the significance of the results of the corresponding mutant x mutant crosses. However, most of the recipient strains produced reasonably large numbers of prototrophs when treated with phage grown on the wild-type strain, thereby allowing the significance of the results of mutant x mutant crosses to be assessed.

Before evaluating the results of independent transduction tests it is necessary to consider a number of points:

(1) The yield of prototrophs may vary from cross to cross as a result of slight differences in the transduction procedure. The extent of this variation can best be judged by repeating crosses which appear to give significant results.

(2) There may be differences in the ease with which different bacterial strains (or even different subcultures of a single strain) are transduced by the same phage. It was not necessary to correct for this variation in the above experiment, because the crosses which had to be compared always involved the same recipient culture.

(3) The recovery of fewer transductants in mutant x mutant crosses than in the corresponding mutant x wild-type crosses may well reflect differences in the efficiency of transduction by different phages rather than linkage of the two markers concerned. Thus in order to compare the results obtained with a single recipient but with different phages, it is necessary to establish a baseline. Several workers (e.g. Ozeki, 1959; Yanofsky and Lennox, 1959) have used an unlinked marker for this standardisation, and so it was decided to look for evidence that one of the markers used in the present experiment was not linked to any of the others. From the general pattern of the results, it seemed likely that the ura marker was a suitable choice, because any phage which was found to give a reduced number of ura$^+$ transductants was found to give reduced numbers with most of the other markers. The figures given in table 3.18 were therefore corrected for differences in the efficiency of transduction by different phages, using the efficiency of transduction of the ura marker as a standard. These corrections would also tend to minimise fluctuations caused by slight differences in the titres of the various phage preparations.

The corrected results are presented in table 3.19, and the figures which are underlined represent mutant x mutant crosses which appeared to yield significantly lower numbers of prototrophs than the corresponding mutant x wild-type crosses.

Table 3.19

Recipient	Donor strains:										
	ade-5	ilva-2	ilva-3	thr-2	thi-2	thi-3	leu-1	leu-2	pro-1	pro-2	wild-type
ade-1	135	55	c	129	611	185	171	266	197	625	675
ade-2	11	15	c	41	81	148	57	46	57	77	116
ade-3	127	200	388	253	302	391	315	191	169	476	625
ade-4	0	25	c	70	0	7	51	14	28	37	71
leu-1	141	0	c	82	85	0	0	0	88	134	330
leu-2	234	40	94	166	268	26	7	4	184	162	246
pro-1	51	85	c	94	115	67	173	95	0	20	61
pro-2	27	78	58	22	151	52	62	69	13	0	173
cys.met	131	90	c	134	390	593	224	105	101	240	293
arg	344	75	c	179	644	89	462	236	353	687	498
lys	513	388	c	427	1072	194	479	212	325	824	730
ura	360	360	360	360	360	360	360	360	360	360	360

c - phage preparation was contaminated

These figures are the total transductants on four plates, corrected for the reverse mutations of the recipient strains, and for differences in the efficiency of transduction by different phages.

Transduction tests with Klebsiella auxotrophs - corrected results

Linkage relationships between selected nutritional markers

In the previous experiment it was found that several of the recipient strains yielded only a very few transductants in mutant x wild-type crosses. The reason for this behaviour was not known, but it seemed likely that better results would be obtained if more concentrated phage preparations were used.

High titre lysates were prepared by growing phage PW52 on each of the ten auxotrophic derivatives of strain W70. Several of the lysates were found to contain 8-10 x 10^{10} p.f.p./ml., and the others were concentrated to this level by centrifugation at 65,000 g for 1 hr. Transduction experiments were then carried out as described above, but this time the recipient cultures were treated with the more concentrated phage preparations.

Results

The crosses which were repeated in this experiment were those which appeared to give significant results in the previous experiment, the reciprocals of those which appeared to give significant results (where possible), and the mutant x wild-type tests. Crosses involving the ura marker were also carried out to provide controls for differences in the efficiency of transduction by different phages, and for slight differences in the titres of the various phage preparations.

From the results which are shown in table 3.20 (the results shown in tables 3.21 - 3.30 are taken from tables 3.19 and 3.20), it can be seen that several mutant x mutant crosses yielded fewer prototrophs than the corresponding mutant x wild-

Table 3.20

Recipient	Donor strains:										
	ade-5	ilva-2	ilva-3	thr-2	thi-2	thi-3	leu-1	leu-2	pro-1	pro-2	wild-type
ade-1	1027				870						1732
ade-2					493						270
ade-3					863						711
ade-4	0				6						208
ade-5	0	130							57	0	288
ilva-1			0	136							393
ilva-2	80	0	108	102		575	194	294	370	142	260
ilva-3		65	0		288		387				95
thr-1				34			21	0		85	294
thr-2		0						42		71	100
thi-1	16										263
thi-2					0	0					191
thi-3					0	0	387				95
leu-1		355	688		318	22					721
leu-2		0									885
pro-1	400	650				88					774
pro-2	160	0		68							260
cys.met											N
met				0							50
arg		390		799		220					1120
lys						198		1176			2095
ura	2280	2280	2280	2280	2280	2280	2280	2280	2280	2280	2280

N = not tested

These figures are the total transductants on four plates, corrected for the reverse mutations of the recipient strain, and for differences in the efficiency of transduction by different phages.

Linkage relationships between selected nutritional markers - transduction tests with high titre phage preparations

Table 3.21

Recipient	Donor	
	ade-5	wild-type
ade-1	135	675
ade-2	11	116
ade-3	127	625
ade-4	0	208
ade-5	0	288

Linkage relationships of ade mutants

Table 3.22

Recipient	Donor		
	thi-2	thi-3	wild-type
thi-1	0	0	88
thi-2	0	0	191
thi-3	0	0	95

Linkage relationships of thi mutants

type crosses. In some cases there was no agreement between the results of reciprocal tests, and in other cases it was found that donor phenotype selection tests (see later experiments) did not provide confirmation of linkage relationships. However, several of the crosses appeared to give significant results, and these will now be discussed in some detail.

(a) Adenine. When the five independently-isolated ade mutants were tested for possible cross-feeding relationships, it was noticed that three of them (ade-3, ade-4 and ade-5) inhibited growth of the other two (ade-1 and ade-2). This result suggested that there were at least two different classes of ade mutants, and that the representatives of one class accumulated a biosynthetic intermediate which was capable of inhibiting representatives of the other class. However, it was found that the ade-3 mutant had only a very slight inhibitory effect, and so it seemed likely that this mutant differed from the ade-4 and ade-5 mutants. Further evidence supporting this view was obtained in the transduction experiments, when it was found that ade-4 x ade-5 crosses yielded no prototrophs at all (suggesting particularly close linkage of these markers) whereas ade-3 x ade-5 crosses yielded significant numbers of prototrophs.

Transduction tests with the five ade mutants were limited to crosses in which the ade-5 derivative was the donor strain, because none of the W52 derivatives (ade-1, ade-2, ade-3 and ade-4) could be used for propagating phage PW52. Thus it would be unwise to draw any firm conclusions about linkage

Table 3.23

Recipient	Donor	
	thi-2	wild-type
ade-1	870	1732
ade-2	493	270
ade-3	863	711
ade-4	6	208

Table 3.24

Recipient	Donor	
	thi-3	wild-type
ade-1	185	675
ade-2	148	116
ade-3	391	625
ade-4	7	71

Table 3.25

Recipient	Donor	
	ade-5	wild-type
thi-1	16	263
thi-3	0	33

Linkage relationships of ade and thi mutants

relationships at this stage, but since each of the ade x ade crosses yielded fewer prototrophs than the corresponding ade x wild-type cross it seems possible that all five markers are linked.

(b) Thiamine (Vitamin B1). In cross-feeding tests, it was found that the thi-1 mutant stimulated growth of the thi-2 and thi-3 mutants. This result suggests that the three mutants can be divided into two groups, but since no prototrophs were recovered in any of the mutant x mutant crosses it seems likely that all three mutational sites are closely adjacent on the chromosome.

(c) Adenine and thiamine. From the results presented in tables 3.23 - 3.25 it can be seen that the ade-4 marker appears to be linked to the thi-2 and thi-3 markers, and the ade-5 marker appears to be linked to the thi-1 and thi-3 markers. On the other hand, the ade-3 marker does not appear to be linked to thi-2 or thi-3. These findings tend to confirm the view that the ade-3 marker represents a mutation at a locus some distance from the closely linked ade-4 and ade-5 markers, and it now seems highly likely that the three thi markers are also closely linked.

(d) Leucine. Cross-feeding tests did not differentiate between the two leu mutants, and the transduction tests suggested that the mutational sites were closely adjacent. It was found that more prototrophs were recovered when strain W70leu-2(PW52) was treated with phage grown on W70leu-1 than were recovered on control plates, but it seems likely that

Table 3.26

Recipient	Donor		
	leu-1	leu-2	wild-type
leu-1	0	0	330
leu-2	7	4	246

Linkage relationships of leu mutants

Table 3.27

Recipient	Donor	
	thr-2	wild-type
thr-1	34	294

Linkage relationships of thr mutants

Table 3.28

Recipient	Donor			
	thr-2	leu-1	leu-2	wild-type
thr-1	34	21	0	294
thr-2	0	-	42	100
leu-1	82	0	0	330
leu-2	166	7	4	246

Linkage relationships of leu and thr mutants

these colonies were revertants because it was also found that more prototrophs were recovered in the self cross (leu-2 x leu-2) than in the control experiment.

(e) Threonine. Cross-feeding tests did not differentiate between the two thr mutants. Fewer transductants were recovered when thr-1 was treated with phage grown on thr-2 than were recovered in the thr-1 x wild-type cross, and so it seems fairly likely that the two markers are linked.

(f) Leucine and threonine. It was not possible to use the thr-1 mutant as a donor strain, and so the results of the crosses involving the thr and leu markers are difficult to assess. However, it seems likely that thr-1 is linked to leu-1 and possibly also to leu-2, while thr-2 may well be linked rather more distantly to leu-2.

(g) Isoleucine and valine (ilva). Three independently isolated mutants were found to require both isoleucine and valine for growth. When these mutants were tested for possible cross-feeding relationships, it was found that the ilva-1 and ilva-3 mutants did not stimulate growth of one another, but did stimulate growth of the ilva-2 mutant. Transduction tests showed that the ilva-1 x ilva-3 cross did not yield any prototrophs; this result suggests that these two strains carry mutations at closely adjacent sites. On the other hand, it seems likely that ilva-2 represents a mutation at a relatively distant site, because the ilva-1 x ilva-2 cross and the reciprocal crosses involving ilva-2 and ilva-3 yielded significant numbers of prototrophs. Thus there is

Table 3.29

Recipient	Donor		
	ilva-2	*ilva*-3	wild-type
ilva-1	136	0	393
ilva-2	0	108	260
ilva-3	65	0	95

Linkage relationships of *ilva* mutants

Table 3.30

Recipient	Donor		
	pro-1	*pro*-2	wild-type
pro-1	0	20	61
pro-2	13	0	173

Linkage relationships of *pro* mutants

good agreement between the cross-feeding and transduction results, and since each *ilva* mutant yielded fewer prototrophs when treated with phage grown on another *ilva* mutant than when treated with phage grown on the wild-type, it seems possible that the three mutational sites are distributed between two linked *ilva* loci. However, several other crosses involving the *ilva*-1 and *ilva*-3 markers yielded reduced numbers of prototrophs, and so no definite conclusions can be drawn at this stage.

(h) *Proline*. Cross-feeding tests did not differentiate between the two *pro* mutants. However, the transduction experiments showed that *pro*-1 x *pro*-2 crosses yielded prototrophic recombinants, and so it appears that the relevant mutations are at different sites. Furthermore, since fewer transductants were observed in *pro* x *pro* crosses than in *pro* x wild-type crosses, it seems likely that the mutational sites are closely adjacent.

Donor phenotype selection tests

Convincing evidence of linkage can often be obtained in donor phenotype selection tests (as described by Clowes, 1958), where phage propagated on one mutant is used to infect bacterial cells of a second mutant and transductants are selected on minimal agar supplemented with the growth requirement of the donor strain. For example, in a cross of *thr*$^-$ (bacteria) with *leu*$^-$ (phage), if *thr* and *leu* are linked, any *thr*$^+$ *leu*$^-$ cells resulting from simultaneous transduction of

the two markers will have the *leu*$^-$ phenotype and will produce colonies on leucine-supplemented minimal agar, on which cells of the recipient *thr*$^-$ strain do not grow. Colonies of this type can be distinguished from the wild-type (*thr*$^+$ *leu*$^+$) by their inability to grow when replicated to unsupplemented minimal agar.

From the above description, it is apparent that donor phenotype selection tests can only be carried out between phenotypically distinct mutants when the donor cells require a supplement which does not support the growth of the recipient cells. Furthermore, the simultaneous transfer of two markers is only expected when the loci concerned are closely linked. However, it had already been found that some 35 crosses between phenotypically distinct mutants yielded fewer prototrophs than the corresponding mutant x wild-type crosses, and since such results provided preliminary evidence of possible linkage relationships it seemed likely that donor phenotype selection tests would be of considerable value at this stage.

In a typical test, phage propagated on one mutant was used to infect cells of a second mutant. Equal samples of the transduction mixture were then plated on minimal agar supplemented with the growth requirement of the donor strain and on unsupplemented minimal agar. The plates were examined for transductants after incubation at 37° for 48 hr. In some cases it was found that more colonies appeared on minimal agar containing the appropriate supplement than appeared on the unsupplemented medium (see table 3.31), and it seemed likely

Table 3.31

Donor	Recipient	Minimal	Minimal + Supplement*	Donor	Recipient	Minimal	Minimal + Supplement*
thi-3	lys	144	155	ade-5	pro-2	48	37
thi-2	ilva-3	445	398	leu-1	ilva-3	130	166
pro-2	ilva-2	68	33	ilva-2	ade-5	6	8
leu-2	ilva-2	99	56	ilva-2	arg	60	58
thi-3	pro-1	35	26	thr-2	ilva-1	22	31
pro-2	ade-5	22	26	ilva-2	thr-2	8	10
thi-3	arg	50	48	pro-1	ade-5	25	21
thi-2	ade-4	130	282	ade-5	pro-1	70	67
pro-2	thr-2	6	5	ilva-3	thi-2	33	15
pro-2	thr-1	54	58	thr-2	ilva-2	90	89
leu-1	thr-1	12	33	leu-1	thi-3	81	132
thi-3	leu-1	48	48	leu-1	ilva-2	223	207
thr-2	met	0	0	thr-2	pro-2	27	34
pro-2	thi-3	88	73	ade-5	thi-1	0	7
ilva-2	leu-2	35	68	pro-1	thi-3	66	66
ilva-2	leu-1	106	118	ilva-2	pro-2	14	16
ade-5	ilva-2	5	0	ilva-3	leu-1	174	125
thr-2	arg	225	235				

* The supplement was in each case the primary growth requirement of the donor strain.

Each figure represents the number of transductants/ml. plated, corrected for the reverse mutations of the recipient strain.

Donor phenotype selection tests

that the excess colonies represented recipient cells which had acquired the donor phenotype as a result of co-transduction of the two markers concerned. To test this suggestion, colonies from appropriate supplemented minimal plates were replicated (with sterile velvet) both to minimal agar and to minimal agar containing the same supplement. It was found that virtually all of the colonies were capable of growing when replicated to minimal agar, but it was felt that this result probably reflected either carry-over of nutrients in the relatively large inocula or impurity of the colonies on the original selective plates. It was therefore decided to repeat the crosses which appeared to give significant results, and to purify the transductants before carrying out further tests.

In the second experiment, six of the mutant x mutant crosses yielded many more transductants when plated on appropriately supplemented minimal agar than when plated on unsupplemented minimal agar. Several well-isolated colonies were picked from each of the supplemented plates, and purified by replating on the same medium. After two successive single colony isolations the sub-lines were tested for ability to grow on minimal agar with and without supplements. From the results (see table 3.32) it can be seen that 17/30 of the transductants arising from the thi-2 x ade-4 cross had acquired the phenotype thi$^-$ ade$^+$, and it seems likely that this phenotype resulted from co-transduction of the thi-2 and ade-4 markers. None of the other crosses were found to yield transductants with a phenotype characteristic of the donor

Table 3.32

Donor	Recipient	No. of clones growing on:			No. having donor phenotype
		Minimal	Minimal + requirement of recipient	Minimal + requirement of donor	
thi-2	ade-4	13/30	13/30	30/30	17/30
leu-1	thr-1	22/22	22/22	22/22	0/22
ilva-2	leu-2	24/24	24/24	24/24	0/24
leu-1	ilva-3	28/28	28/28	28/28	0/28
leu-1	thi-3	29/30	29/30	29/30	0/30
ade-5	thi-1	7/7	7/7	7/7	0/7

Determination of phenotype of purified clones emerging from transduction experiments

strain, but this may well have been because the colonies on the original selection plates were impure and it was only possible to test a relatively small number of purified sub-lines.

SECTION IV

CONJUGATION

Introduction

Conjugation has already been defined as a mechanism of genetic transfer which requires cellular contact between donor and recipient bacteria. The phenomenon was first described in E. coli K12 (Lederberg and Tatum, 1946), and has since been found to occur in several other bacterial species. Only the E. coli K12 system has been extensively analysed, but there appear to be strong similarities between this system and the others.

Conjugating strains are normally differentiated into donor (male) and recipient (female) types, although it should perhaps be noted that there is as yet no evidence of sexual differentiation in the Pseudomonas echinoides system (Heumann, 1962). In most of the other known conjugation systems, the ability to conjugate is conferred upon cells by the presence of discrete genetic determinants, which may exist independently of the chromosome and be transferred with high frequency during conjugation. Thus it is possible that the crosses described by Heumann (1962) are all of the $F^+ \times F^+$ type (i.e. the conjugation determinant is present in both partners); evidence of differentiation will not therefore be available until the equivalent of an F^- strain is found. Several different types of conjugation determinant have been described, and it has been demonstrated that some, if not all, are capable of existing in two alternative states: an autonomous state, in which they are transferred independently of the bacterial chromosome, and an

integrated state, in which they are transferred along with it. Of these determinants, the F factor of E. coli K12 has been most extensively studied, and so some of the properties associated with this factor will be briefly described.

When E. coli K12 cells become infected with the F factor, they are found to differ from uninfected cells in several respects. The most obvious difference is that the cells become capable of forming mating unions with recipient cells, and may act either as F^+ or Hfr donors. Several lines of evidence show that the ability to form unions is due to the synthesis of a new surface component:

(i) donor cells have greater precipitability at low pH values (Maccacaro, 1955), different affinity for certain dyes (Maccacaro and Comolli, 1956), and a different electrophoretic mobility (Turri and Maccacaro, 1960);

(ii) donor cells possess a surface antigen, the f^+ antigen, which recipient cells do not have (Ørskov and Ørskov, 1960);

(iii) the ability of donor bacteria to mate can be temporarily abolished by treating them with periodate, and mating capacity can then be restored by growth in the absence of periodate (Sneath and Lederberg, 1961);

(iv) donor cells are sensitive to a group of RNA phages to which recipient cells are resistant (Loeb, 1960; Loeb and Zinder, 1961; Dettori, Maccacaro and Piccinin, 1961).

Periodate treatment also removes the receptors for the RNA phages (Dettori et al., 1961), so that the surface

property responsible for mating and for adsorption of these donor specific phages is probably the same (Hayes, 1964). Moreover, the receptor for these phages appears to be a specific type of surface appendage, called the F pilus (Crawford and Gesteland, 1964), and recent studies by Ishibashi (1967) suggest the identity of F pili and the f^+ antigen of donor cells. Brinton (1965) has argued plausibly and convincingly that F pili are the actual organs of genetic transfer; the evidence is largely circumstantial, and requires a lengthy exposition which is not possible here. Very briefly, the main points of evidence are:

(i) it has been shown that the F factor itself controls the synthesis of F pili;

(ii) genetic or phenotypic loss of F pili results in loss of donor ability, and donor ability is regained at the same time and the same rate as F pili when genetic manipulation or cultural conditions allow the gain of F pili;

(iii) F pili can conduct the RNA and probably the DNA of male specific phages into the male cell, and the fine structure of the F pilus appears admirably suited to the conduction of long molecules of nucleic acid since it can be seen to have an axial hole 20 to 25A^o in diameter.

An earlier theory implied that genetic transfer occurred through electron-microscopically visible intercytoplasmic connections called conjugation bridges, but Brinton (1965) has produced evidence which suggests that these "bridges" are artifacts unconnected with genetic transfer phenomena. It

seems likely, therefore, that F pili provide an entry channel through the cytoplasmic membrane for the RNA and possibly DNA of male specific phages, and in conjugation F pili may act as chennelling devices for bacterial DNA. Further support for this idea has been obtained recently by a number of workers, including Knolle (1967) who found that the conjugation process effectively reduces the efficiency of infection by RNA phages, while affecting adsorption to a much lesser extent. Thus F pili may well be the male sex organs of bacteria, but very little is known about the events which lead to the formation of the union between a male and a female cell, apart from a suggestion that male cells may synthesise an enzyme which mediates penetration of the female cell (Clowes and Moody, quoted in Hayes, 1966a).

It seems likely that a male cell carries only one F pilus per sex factor (Brinton, 1965), and the question now arises: how does the sex factor "find" the F pilus through which it is supposed to pass? One popular answer is that the sex factor (either in the autonomous or integrated state) is attached to the cell membrane and makes its antigen locally, so that it is at this point that the tube or pilus is formed (Jacob, Brenner and Cuzin, 1963; Jacob, 1966). Furthermore, Jacob et al. (1963) have proposed a general model of the sex factor/chromosome interactions which might lead to transfer. This model is based on the hypothesis that the chromosomes of bacteria and sex factors are circular and constitute units of replication or "replicons". Each replicon controls its own

replication, carrying a locus which is activated by a specific cytoplasmic initiator so that it opens up at the locus to form a template for the polarised synthesis of a new replicon. It is now suggested that a surface reaction which occurs while the male and female come in contact triggers, in some way, a round of replication in the male. One of the structures thus synthesised remains in the male, while the other is transferred, during its formation, to the female (Gross and Caro, 1965; Ptashne, 1965). If the sex factor is autonomous, it would be expected to replicate and be transferred as an independent structure, whereas if the sex factor is integrated, the replica would be continued around the bacterial chromosome and transfer of the whole structure would be initiated. A number of workers have obtained critical evidence bearing on this model (discussed by Gross, 1965, and Hayes, 1966b), but not all of the findings are favourable, and no firm conclusions can be drawn at present.

Most of the studies concerned with the mechanism of genetic transfer during conjugation have involved crosses between F^+ and F^-, Hfr and F^-, and F-prime and F^- strains of E. coli K12. However, it seems possible that several other bacterial genetic elements have analagous properties (Jacob, 1966), and indeed the F factor itself may behave in much the same way whether it is contained in E. coli K12 cells or in cells of certain other species. For example, the F factor has been transferred to several different strains of E. coli, and to strains of Salmonella and Shigella (Bernstein, 1958;

Zinder, 1960b; Luria and Burrous, 1957). The F^+ strains so obtained may be able to transfer F back to E. coli K12, and in some cases may be able to act as donors of chromosomal genetic material. The yield of recombinants in F^+ by F^- crosses in Salmonella is similar to that in E. coli (Zinder, 1960b), but on the other hand F^+ Shigella strains do not appear to give recombinants in crosses with other Shigella derivatives or with F^- strains of E. coli K12 (Luria and Burrous, 1957). To explain this finding, Luria and Burrous (1957) have suggested that the Shigella chromosome cannot be mobilised for transfer because it lacks regions of homology to which the sex factor can attach.

When the F factor is transferred to certain Salmonella strains, it may be possible to isolate stable Hfr Salmonella donors which can transfer segments of their chromosome to Salmonella, Shigella and E. coli recipients (Baron, Carey and Spilman, 1959; Zinder, 1960b). However, crosses between Hfr donor strains of E. coli K12 and Salmonella recipients are usually infertile, although in a few cases low yields of recombinants can be obtained (Baron, Spilman and Carey, 1959; Miyake and Demerec, 1959; Zinder, 1960a). It seems that genetic homology is important in the formation of recombinants, because some Salmonella strains which do not yield any chromosomal recombinants can nevertheless act as recipients of F' factors (Ørskov, Ørskov and Kauffmann, 1961). Other bacteria which can accept F' factors from E. coli K12 include strains of Serratia marcescens, Vibrio cholerae, Proteus mirabilis

(Baron, 1965), and Pasteurella pestis (Martin and Jacob, 1962). Many of these strains are known to possess DNA base ratios which differ markedly from the base ratios of E. coli strains; this probably reflects a lack of genetic homology which in turn prevents recombination with chromosomal genetic material from an E. coli donor.

It seems likely therefore that F' factors (e.g. F-lac) can establish themselves in "foreign" cellular environments simply because they are able to persist in the unintegrated (autonomous) state (Marmur et al., 1963). Once present in a foreign cell, F or F' factors may be able to mediate their own transfer to homologous recipients, or to F^- E. coli strains, but in most cases crosses of this type do not yield chromosomal recombinants. The explanation adopted by Luria and Burrous (1957) for their Shigella $F^+ \times F^-$ system may also apply here: the "foreign" chromosomes may not be mobilised for transfer because they lack regions of homology to which the E. coli sex factor can attach. However, if chromosomal transfer can be initiated, and if there are derivatives which are capable of acting as recipients, intrastrain crosses should yield recombinants. On the other hand, interstrain or intergeneric crosses may not give recombinants unless the transferred genetic material has regions of homology which will allow integration and recombination with the recipient chromosome. Furthermore, even when there are regions of homology, it is believed that transferred DNA may sometimes be recognised as foreign and broken down before integration can occur (Dussoix

and Arber, 1962; Boyer, 1964; Rolfe and Holloway, 1966). The precise nature of this restriction mechanism is not clear, but presumably a specific nuclease capable of recognising foreign DNA is involved. Boyer (1964) has suggested that it might be possible to overcome the restriction mechanism of recipient cells if one can introduce large fragments of male DNA which could saturate the cell nucleases, and Rolfe and Holloway (1966) have produced evidence to show that Pseudomonas aeruginosa restriction mechanisms can be altered by growth of the recipient strain at 43° instead of at 37°.

A few attempts have been made to study interactions between F factors and Klebsiella strains. Makela, Lederberg and Lederberg (1962) and de Haan, Stouthamer, Felix and Mol (1963) have reported that several Klebsiella strains can act as recipients of F-prime factors, but other workers have been unable to detect recombinants in crosses between Hfr strains of E. coli K12 and many different Klebsiella strains (Clarke, 1960, 1961; Ørskov and Ørskov, 1961; de Haan et al., 1963). Most of these studies were basically concerned with other topics, and as a result the Klebsiella experiments are described only in outline. Apart from two strains mentioned by Clarke (1960), it is not clear whether any non-capsulate Klebsiella mutants were used as potential recipients. If not, many of the difficulties which were experienced may be explicable, because it is to be expected that occlusion of the cell surface by a polysaccharide capsule might seriously interfere with the formation of mating unions. Another important

point is that de Haan et al. (1963) were unable to detect transfer of F' factors to Klebsiella strains in broth or on membrane filters, but were successful when donor and acceptor bacteria were plated together on a selective medium; it was concluded that contact formation was a very important factor in such crosses. Thus the experiments of Clarke (1960, 1961) and Ørskov and Ørskov (1961) may have been unsuccessful primarily because their Hfr E. coli donors and Klebsiella recipients were unable to form effective contacts in mixed broth culture, but even if this difficulty can be overcome there may still be problems of genetic homology and restriction mechanisms which will have to be considered.

Colicinogenic and bacteriocinogenic factors

Bacteriocins are protein or protein-containing antibiotics which are produced by various species of bacteria, and which are generally active only on strains of the same or closely related species. The first bacteriocins to be studied in detail, the colicins, are produced by some strains of Enterobacteriaceae and are active on other members of the family. Colicin-producing (colicinogenic) bacteria may carry the determinants of more than one colicin; different colicins, which are designated by capital letters (e.g. I, V, E1, E2), may be distinguished by their diffusibility and host specificity (Hayes, 1964).

The determinants of certain colicins (col factors) can be transmitted from col^+ to col^- bacteria by cellular contact

between strains of the same species, or between strains of Salmonella, Shigella and Escherichia (Frédéricq, 1957). In some cases, col factor transfer appears to be a consequence of F-mediated conjugation, since it occurs only between F^+col^+ and F^-col^- cells (Frédéricq, 1954). For example, in F^+col El x F^-col^- crosses, transfer of col El proceeds efficiently and is not associated with the transfer of any other donor marker except the sex factor (Frédéricq, 1958). From this and other evidence it has been suggested that the col El determinant behaves as an autonomous cytoplasmic element (see Frédéricq, 1965). However, certain other col factors (e.g. col I) strongly resemble the F factor, since they are able to mediate their own transmission in F^-col^+ x F^-col^- crosses (Clowes, 1961). A further resemblance to the F factor became apparent when it was found that S. typhimurium cells which had recently acquired col I could bring about conjugal transfer of chromosomal determinants with low frequency (Ozeki and Howarth, 1961). Similar low frequency chromosome transfer has been observed in E. coli K12 (Clowes, 1961).

An interesting feature of colicinogeny transfer is that many established col^+ strains transmit their col factor at a very low rate, whereas bacteria which have just acquired a col factor transmit it with great efficiency (Stocker, Smith and Ozeki, 1963). For this reason, a special technique is used to obtain high-frequency transmission of colicinogeny: broth is inoculated with cells of col^+ and col^- strains, incubated overnight at 37^o, diluted tenfold in fresh broth, and incubated

for a further two hours. In an experiment of this type, it is likely that a high proportion of the col$^-$ cells will acquire the col factor within the final two hours' incubation, and will therefore be able to transmit the factor very efficiently. For example, high-frequency colicinogeny transfer (HFCT) cultures of an S. typhimurium strain were obtained in this way, and it was found that such cultures were able to transmit col I to about 50% of col$^-$ bacteria within one hour, whereas established col I strains normally transmit the factor to less than 0.5% of the recipient bacteria in five hours (Smith and Stocker, 1962).

When two genetically marked S. typhimurium strains are crossed by mixing an HFCT col I culture of one with a col$^-$ culture of the other, chromosomal recombinants may be detected. Furthermore, if the HFCT donor carries the col E1 factor as well as the newly acquired col I factor, the frequency of recombination may be enhanced 100-fold (Ozeki and Howarth, 1961). Thus the col E1 factor, which does not by itself permit transmission or recombination, must play some as yet undefined role in promoting transfer of the chromosome (Ozeki and Howarth, 1961; Hayes, 1964). Studies of this type have been of value in over-all mapping of the S. typhimurium chromosome (Smith and Stocker, 1962), and may well prove useful in dealing with other bacterial groups.

Bacteriocinogeny is known to occur in many other genera, including Vibrio (Farkas-Himsley and Seyfried, 1963), Pasteurella, Serratia, Hafnia, Erwinia, Enterobacter

(Aerobacter), Klebsiella, Listeria, Streptococcus, Staphylococcus and Bacillus (Reeves, 1965). It is possible that in many cases bacteriocinogeny depends upon the presence of transmissible plasmids, but the available evidence relates only to col factors and one or two others. However, the fertility system in Vibrio cholerae is probably mediated by a bacteriocin factor, the P factor (Bhaskaran, 1960, 1964), and other such systems may well be found in the future.

In studies with Klebsiella, there are two possible approaches. Firstly, Hamon (1956) has shown that colicinogeny can be transferred from one particular E. coli strain (K 30) to two Klebsiella strains, and there is therefore a possibility that col factors may be able to mediate conjugation in Klebsiella. Secondly, several workers have described bacteriocinogenic Klebsiella strains (Hamon and Peron, 1963; Durlakowa, Maresz-Babczyszyn, Przondo-Hessek, Lusar and Mroz-Kurpiela, 1964a and b; Stouthamer and Tieze, 1966), and, as mentioned in the introduction to the chapter on transduction, some of the strains described by Ciuca et al. (1959) and Eustatziou et al. (1960, 1962) may well be bacteriocinogenic rather than lysogenic. Some of these bacteriocinogenic strains may themselves carry factors which can mediate conjugation.

Transmissible drug resistance

Resistance factors (R factors) are extra-chromosomal genetic elements which render their bacterial hosts resistant

to one or more antibacterial agents. They are able, like the sex factor, F, to promote conjugation between bacterial strains. Originally described in shigellae in Japan, R factors have now been found by a number of workers in strains isolated in Europe (Datta, 1965) and in the United States (Smith, 1966). Many of the early studies of the nature and incidence of R factors were carried out in Japan and were reported in Japanese, but several excellent and detailed reviews have appeared in English (Watanabe, 1963a, 1964, 1966).

It has been shown that intact cells of resistant donors are essential for transfer of R factors, except in rather unusual instances in which transduction occurs (Watanabe and Fukasawa, 1961a; Watanabe, 1964). However, since neither phage nor F factor is required, it seems that the R factor itself is able to bring about conjugation and transfer (Mitsuhashi, Harada and Hashimoto, 1960). The hypothetical part of the R factor which mediates conjugation and transfer has been called the resistance transfer factor (RTF or RT factor), and an R factor may therefore be considered as a genetic element which consists of an RT factor and a variable number of drug resistance determinants.

A large group of R factors have been found to have the capacity to inhibit the function of the F factor when introduced into F^+ bacteria; such factors are known as fi^+ (for fertility inhibition: Watanabe, Nishida, Ogata, Arai and Sato, 1964). These factors closely resemble F in that $R^+fi^+F^-$ bacteria may, under special conditions, produce the specific F

pilus and thus become sensitive to male specific (or more correctly now, F specific) phages (Meynell and Datta, 1966a). Indeed, a considerable body of evidence has been presented to show that fi^+ factors behave as if they differ from F only in producing a repressor which limits synthesis of the F pilus, whether by the RT factor itself or by F when it is in the same cell (Datta, Lawn and Meynell, 1966; Meynell and Datta, 1966a; Meynell and Datta, 1967).

It has been shown that bacteria which have newly received an R factor transmit it with greater efficiency than bacteria in which it has been established for many generations (Watanabe, 1963b). This phenomenon is called high-frequency resistance transfer (HFRT), and closely resembles high-frequency colicinogeny transfer (HFCT); the most likely explanation is that in both cases transfer to a recipient without preformed repressor is followed by an interval in which conjugating function is uninhibited until a repressor gene acquired with the R or col factor has time to restore the repression (Monk and Clowes, 1964; Meynell and Datta, 1966a). Moreover, the conjugation determinants of certain col factors (e.g. col V2, col V3, and some col B factors) closely resemble those of fi^+ R factors, and differ from F only in producing a repressor of F pili synthesis (Meynell and Datta, 1966b). Thus these col factors and fi^+ R factors may carry identical transfer factors (TFs), in one case linked to genes specifying colicinogeny and in the other case linked to genes specifying drug resistance. However, the conjugation determinants

carried by certain other col factors (e.g. col I and a particular col B factor) and by fi^- R factors do not specify the production of an F type pilus and therefore appear to be unrelated to F (Meynell and Datta, 1966b). Even so, the idea that specialised pili are associated with conjugation does not have to be abandoned, because unusual types of pili ("sex pili") have been observed on cells carrying factors of this type (Meynell and Lawn, 1967; Meynell and Datta, 1967).

Some R factors, like some col factors, give chromosomal recombinants in R^+F^- x F^- crosses (Sugino and Hirota, 1962). The frequency of recombination is normally very low but higher frequencies have been obtained by introducing an R factor into a particular F^- E. coli K12 strain which has a chromosomal locus with a high affinity for F and apparently also for R (Richter, 1961; Sugino and Hirota, 1962). Also, very recently, E. Meynell and N. Datta (personal communication) have isolated strains carrying R factors whose conjugation functions are no longer repressed ("depressed mutants"), and it has been found that these mutants allow relatively high frequencies of chromosomal recombination to be achieved.

R factors can be transferred by mixed cultivation to all genera of the Enterobacteriaceae (Harada, Suzuki, Kameda and Mitsuhashi, 1960; Nakaya, Nakamura and Murata, 1960), and to several other genera of gram negative bacteria (Datta, 1965). Kasuya (1964) has demonstrated that R factors can be transferred from Shigella flexneri or E. coli to K. pneumoniae when mixed cultures are inoculated into germ free mice, and so it

is not surprising that a number of Klebsiella strains isolated from clinical sources have been found to carry R factors (Datta, personal communication; Smith and Armour, 1966; Mitsuhashi, Hashimoto, Egawa, Tanaka and Nagai, 1967). In one rather unusual case, a so-called "spontaneous spheroplast" strain of K. pneumoniae has been shown to carry a non-chromosomal genetic element which resembles an R factor but carries resistance to phage T1 as well as drug resistance markers (Molina, 1964). This genetic element could be transferred by conjugation to E. coli K12, but transfer to other Klebsiella strains was not reported (Molina, 1964; Molina, Calegari and Monti-Bragadin, 1965). However, when the present study was initiated it had been shown that Klebsiella strains could act as recipients of R factors in crosses with Shigella or E. coli donors (Harada et al., 1960; Nakaya et al., 1960); it therefore seemed likely that it would be possible to study crosses in which both donor and recipient were Klebsiella derivatives.

Methods

Testing for bacteriocin production - soft agar overlay method (Frédéricq, 1957)

The prospective bacteriocinogenic cultures were stabbed into nutrient agar plates and incubated at 37° for 48 hr. The macrocolonies which developed were sterilised by exposure to chloroform vapour, and then the plates were overlaid with 5 ml. of molten soft agar (at 46°) containing 0.1 ml. of an overnight broth culture of the sensitive indicator strain. Zones of inhibition of growth surrounding bacteriocinogenic colonies were scored after overnight incubation at 37°.

In certain experiments, 0.1 ml. aliquots of appropriate dilutions of the prospective bacteriocinogenic culture were spread on nutrient agar (or streptomycin nutrient agar) and incubated at 37° for 48 hr. Plates which contained 8-15 well-isolated colonies were then selected and overlaid with seeded soft agar as described above.

Membrane filter mating technique (Matney and Achenbach, 1962)

Nutrient broth cultures of the parental strains were grown overnight at 37°, diluted 1/20 in fresh broth, and incubated at 37° for a further 2 hr. The cells from 4.5 ml. samples of the recipient culture and 0.5 ml. samples of the donor culture were then impinged together and separately on membrane filters (0.45 μ Millipore filters). Each membrane was removed from the filter device with sterile forceps, and

placed on the surface of a plate which contained either nutrient agar or soft minimal agar supplemented with 10 µg. of vitamin B1 per ml. of medium. After incubation for a suitable period to allow mating (usually 2-3 hr. at 37°), the cells were recovered from the membranes by vigorous shaking in saline and plated on a suitable selective medium. Recombinants were scored after incubation at 37° for 1-5 days.

Experiments and Results

Mixed cultivation experiments with Klebsiella auxotrophs

A number of auxotrophic mutants of *K. aerogenes* strain A3 were isolated for use in mixed cultivation experiments. Two auxotrophs derived from *K. pneumoniae* strain 1.9 were also included in this series of experiments.

Aliquots (2 ml.) of overnight broth cultures of the various auxotrophs were mixed in pairs in sterile test tubes. The mixtures, along with controls of the auxotrophs alone, were incubated in a water bath at 37° for 2 hr. After this time, 0.2 ml. samples were removed from each tube and spread on minimal agar to select prototrophic recombinants. The remaining portions of the mixtures and controls were re-incubated at 37° for 16 hr., and then further samples were withdrawn and plated on minimal agar.

In a second series of experiments, overnight broth cultures of the various auxotrophs were centrifuged, and the cells were washed twice in saline. The auxotrophs were then resuspended in saline, and aliquots of the suspensions (containing c. 1×10^{8} cells) were spread individually and in pairs on minimal agar plates. Prototrophic colonies were scored after incubation at 37° for 5 days.

Results

The results are presented in tables 4.1 and 4.2. No evidence of recombination was obtained in any mixed cultivation experiment.

Table 4.1

	1.9/R6	1.9(0)/2	A3(0)his⁻	KP129/S1	KP129/S2	KP105/S1	KP105/S2
1.9/R6		-	-	-	N	N	N
1.9(0)/2			-	-	N	N	N
A3(0)his⁻				-	-	-	-
KP129/S1					-	-	-
KP129/S2						-	-
KP105/S1							-
KP105/S2							

Table 4.2

	A3/S1	A3/S2	A3/S3	A3/S4
A3/S1		-	-	-
A3/S2			-	-
A3/S3				-
A3/S4				

N = not tested.

- = no more prototrophs on mixture plates than on plates spread with the auxotrophs separately.

Mixed cultivation experiments with Klebsiella auxotrophs

Attempted transfer of F-*lac* to a derivative of *K. aerogenes* strain A3 in broth

It has been reported that F' factors can be transferred from *Salmonella abony* or *E. coli* K12 to Klebsiella (Makela *et al.*, 1962; de Haan *et al.*, 1963). The recipient Klebsiella strains were not described in any detail, and may or may not have been capsulate. It was therefore decided to attempt to transfer the F-*lac* factor to a non-capsulate derivative of *K. aerogenes* A3. The characteristics of the various strains used in this experiment are shown in table 4.3.

An overnight broth culture of the donor strain (240) was diluted 1/20 in fresh broth and incubated for a further 2 hr. at 37°. Aliquots (0.2 ml.) of this culture were then mixed with 1.8 ml. aliquots of an overnight broth culture of strain W60, and the mixtures were incubated at 37° for 2 hr. After this time, samples were withdrawn and plated on M9 minimal-lactose agar containing 1% (v/v) nutrient broth, histidine (10 µg./ml.) and streptomycin, to select *lac*$^+$ *str*^{r} recombinants. Samples of the individual parent cultures were plated on the same medium to provide controls for spontaneous mutants.

In a further experiment, 1 ml. aliquots of overnight broth cultures of strains 240 and W60 were mixed in 5 ml. portions of fresh broth and incubated at 37° for 16 hr. before samples were plated on M9 minimal-lactose agar containing 1% (v/v) broth, histidine and streptomycin.

Results

No evidence was found to suggest that F-*lac* could be

transferred to a non-capsulate Klebsiella strain in broth. Makela *et al.* (1962) found that a Klebsiella recipient acquired the F'_{13} factor after prolonged incubation in broth with an *E. coli* or *S. abony* donor strain, but the actual transfer may well have occurred on the surface of the eosin methylene blue minimal-lactose agar medium which they used for selection. This view is supported by the fact that de Haan *et al.* (1963) were also unable to detect transfer of an F' factor to Klebsiella recipients in broth.

Transfer of F-*lac* to a derivative of *K. aerogenes* strain A3

Overnight broth cultures of strains 240 and W60 were centrifuged, and the cells were resuspended in saline. Aliquots of the suspensions containing approximately 2×10^8 cells were then spread separately and together on plates of M9 minimal-lactose agar containing 1% (v/v) broth and histidine. Colonies were scored after incubation at 37° for 2-5 days.

Results

The results are presented in table 4.3. The colonies which appeared on the mixture plates were streaked out on streptomycin-EMB-lactose agar, and two different patterns of growth were observed. One type of colony produced *lac*$^+$ clones exclusively, and it was presumed that these were stable revertants. The other type produced a very few *lac*$^+$ clones in the well of the plate, and *lac*$^-$ clones elsewhere. It seemed possible that a proportion of the cells in this type of colony were *lac*$^+$ derivatives of strain W60. This suggestion

Table 4.3

	No. of colonies/plate on singly-enriched M9-minimal + lactose + histidine*
E. coli strain 240: F-*lac*$^+$*met*$^-$*str*^{s}	0
K. aerogenes strain W60: A3(0)*his*$^-$*lac*$^-$*str*^{r}	4
240 x W60 cross	9

* Average of 12 plates

Transfer of F-*lac* to a derivative of *K. aerogenes* strain A3

was partly confirmed by the finding that the *lac*$^+$ clones were unstable, yielding *lac*$^-$ colonies at a high rate when sub-cultured to fresh plates of streptomycin-EMB-lactose agar. Several attempts were then made to transfer F-*lac* from cultures derived from unstable *lac*$^+$ clones to *E. coli* strain 242 (F$^-$ *pro*$^-$ *thi*$^-$ *lac*$^-$ *str*^{r}) on M9 minimal-lactose agar containing proline and vitamin B1, but no positive results were obtained. De Haan *et al.* (1963) were also unable to detect transfer of the episome from F' Klebsiella strain to other strains of Klebsiella or to *E. coli* K12.

From the above results, it did not seem likely that F-*lac* transfer would provide a good system for study of conjugation in Klebsiella, even when non-capsulate recipients were used, and so no further experiments of this type were carried out.

Attempted transfer of the F factor from *E. coli* K12 to Klebsiella strains

An overnight broth culture of *E. coli* strain W1655 (F$^+$ *met*$^-$ *str*^{s}) was diluted 1/20 in fresh broth and incubated at 37^o for a further 2 hr. Aliquots (0.2 ml.) of the resulting culture were then mixed with 1.8 ml. aliquots of overnight broth cultures of the Klebsiella auxotrophs A3(0)*his*$^-$ *str*^{r}, KP105/S1.*str*^{r}, KP105/S2.*str*^{r}, and KP105/S3.*str*^{r}. The mixtures were incubated at 37^o for 2 hr., and after this time samples were withdrawn and plated on streptomycin-minimal agar. The remaining portions of the mixtures were diluted 1/5 in fresh broth and incubated at 37^o for a further 16 hr. before

samples were plated on streptomycin-minimal agar. Cultures of the individual strains were plated on the same medium to provide controls for spontaneous mutants.

In a second experiment, samples were withdrawn from the W1655F$^+$ *met*$^-$ *str*^{s} x A3(0)*his*$^-$ *str*^{r} cross after 18 hr. incubation, diluted appropriately, and plated on a medium consisting of minimal A + streptomycin + histidine.

Results

No more streptomycin resistant prototrophs were recovered from cultures that had been incubated with the F$^+$ strain than were recovered from control cultures. There are several possible reasons for this result. Firstly, the formation of mating pairs may have been relatively inefficient, a suggestion which is supported by the finding that F' factors can be transferred to Klebsiella recipients on the surface of a solid medium but probably not in broth (de Haan *et al.*, 1963). Secondly, any DNA transferred from the *E. coli* donor may have been recognised as "foreign" and immediately rejected or broken down by the Klebsiella recipients. Thirdly, the Klebsiella recipients may have been incapable of incorporating fragments of the *E. coli* chromosome into their own genomes, in which case stable (colony-forming) recombinants would not have been detected.

In the second experiment, 50 of the colonies which appeared on the minimal + streptomycin + histidine plates were purified by re-streaking on the same medium. Single well-isolated A3(0)*his*$^-$ *str*^{r} colonies were then picked from the

purification plates, inoculated into broth tubes, and incubated at 37° overnight. The resulting cultures were used to seed soft agar overlays and spot tested for sensitivity to the male-specific phage MS-2. None of the cultures were found to be sensitive to MS-2, and so it was not possible to demonstrate the presence of the F factor in cells which had been incubated with the F^+ E. coli strain.

Attempts to detect F-mediated transfer between Klebsiella strains

Another possible way of detecting transfer of the F factor to Klebsiella strains was briefly investigated. This method depended on (1) transfer of the F factor from E. coli K12 to a prototrophic Klebsiella strain, and (2) F-mediated chromosomal transfer from infected recipient cells to a Klebsiella auxotroph. The F^+ strain and the prototroph were sensitive to streptomycin whereas the auxotroph was resistant, and so selection for recombinants could be accomplished by plating on streptomycin-minimal agar.

An overnight broth culture of strain W1655(F^+ met^- str^s) was diluted 1/20 in fresh broth and incubated at 37° for a further 2 hr. A 0.2 ml. aliquot of this culture was then added to a tube which contained 1 ml. of sterile broth and a 1.8 ml. aliquot of an overnight broth culture of strain KP105 str^s. The mixture was incubated at 37° for 2 hr., and after this time a 2 ml. aliquot of a 22 hr. broth culture of strain A3(0)his^- str^r was added. The triple culture was incubated

at 37°, and samples were withdrawn after 2 hr. and 16 hr. for plating on streptomycin-minimal agar. Controls of the recipient alone, and of the donor mixture alone, were plated on the same medium.

Results

No more streptomycin resistant prototrophs were recovered from the triple culture than were recovered from control cultures, and so F-mediated transfer between Klebsiella strains was not observed.

E. coli Hfr x Klebsiella crosses

Attempts were made to detect transfer of the str^r marker from an E. coli K12 donor strain to various streptomycin sensitive Klebsiella recipients. The donor strain was S56 (thi^- lac^- thr^- leu^- str^r), an Hfr derivative which transfers the str^r marker early during conjugation, and the recipients were six independently-isolated non-capsulate mutants of K. aerogenes strain A3 (A3(0), KP105, KP109, KP114, KP120 and KP130). An F^- E. coli K12 recipient was included in this experiment to provide a positive control. Selection for recipients which had acquired the str^r marker was accomplished by plating on streptomycin-minimal agar.

Donor cultures were prepared by diluting overnight broth cultures of strain S56 1/20 in fresh broth and re-incubating at 37° for 2 hr. A typical cross was carried out by mixing a 0.1 ml. portion of a donor culture with 1 ml. of sterile broth and 1.9 ml. of an 18 hr., 37° culture of the appropriate

recipient strain. The mixture was incubated at 37° for 3 hr., and then 0.2 ml. samples were withdrawn and plated on streptomycin-minimal agar. Further samples were withdrawn and plated on the same medium to provide controls for spontaneous mutants.

In a second experiment, the recipient bacteria were incubated in broth at 43° for 24 hr. before crosses were carried out. This procedure has been found to increase recombination frequency between certain Pseudomonas aeruginosa strains in circumstances which suggest that incubation at 43° somehow prevents the breakdown of "foreign" DNA (Rolfe and Holloway, 1966). It seemed highly probable that DNA from E. coli K12 would be recognised as "foreign" in Klebsiella cells, and it was therefore hoped that prior incubation at 43° would allow some recombination to occur.

In a further experiment, donor and recipient bacteria were impinged separately and together on membrane filters as previously described. The impinged cells were incubated at 37° for 2 hr. on minimal agar + vitamin B1 (10 μg./ml.) and then transferred to nutrient agar for a further 4 hr. incubation to allow phenotypic expression of streptomycin resistance (Watanabe and Watanabe, 1959). The cells were recovered from the membranes by vigorous shaking in saline, and aliquots of the resulting suspensions were plated on streptomycin-minimal agar.

Results

Streptomycin resistant prototrophs were recovered in all

S56 x *E. coli* strain 703 (F^-) crosses, but transfer of the str^r marker to Klebsiella recipients was not observed.

Treatment of a bacteriocinogenic Klebsiella strain with acridine dyes

K. pneumoniae strain 1.2 is a bacteriocinogenic, producing an inhibitory agent which is active on certain other Klebsiella strains (Clarke and MacPhee, 1965). As a preliminary to testing this strain for ability to mediate conjugation, attempts were made to demonstrate elimination of the genetic determinant of bacteriocinogeny by treatment with acridine dyes.

Aliquots of an overnight broth culture of strain 1.2 were diluted to 10^4 cells/ml. in portions of nutrient broth (pH 7.6) containing various concentrations of acridine dyes. The resulting cultures were incubated overnight at 37°, and then proper dilutions were spread on nutrient agar plates. After incubation at 37° for 48 hr., plates which had suitable numbers of well-isolated colonies were selected. The colonies on these plates were killed by exposure to chloroform vapour, and then tested for bacteriocin production by the addition of soft agar overlays seeded with the sensitive indicator strain 1.9.

Results

Strain 1.2 was treated with acridine orange at concentrations of 20, 50 and 100 µg./ml., and with 50 and 250 µg./ml. of acriflavine. About 200 colonies were tested for bacteriocin production after each treatment, but it was found that all such colonies remained bacteriocinogenic. Thus although it

has been shown that certain non-chromosomal genetic determinants can be eliminated from bacterial cells by treatment with acridine dyes (e.g. F factors: Hirota, 1960; R factors: Watanabe and Fukasawa, 1961b), it appears that the bacteriocin determinant of strain 1.2 is not susceptible to elimination in this way. This does not necessarily mean that the bacteriocin determinant of strain 1.2 is located on the chromosome, however, because certain other factors which are generally believed to be non-chromosomal do not appear to be eliminated by the standard treatment with acridine dyes (e.g. col factors: Clowes, 1965; FP factor of Pseudomonas aeruginosa: Holloway and Fargie, 1960).

Attempted transfer of the genetic determinant of bacteriocinogeny from K. pneumoniae strain 1.2 to other Klebsiella strains

Three different methods were used in attempts to show that the genetic determinant of bacteriocinogeny could be transferred from strain 1.2 (str^s) to certain other bacterial strains. The prospective recipient strains were all resistant to streptomycin, and so transfer could be detected by plating mixed cultures on streptomycin agar and testing the resulting colonies for bacteriocin production.

(1) Mixed cultivation in broth. A 10 ml. aliquot of broth in a 100 ml. Erlenmeyer flask was seeded with 1 ml. of an overnight broth culture of strain 1.2, and with a similar inoculum from a culture of a recipient strain (A3str^r, KP105str^r or 1.9 str^r). The mixture was incubated overnight,

without shaking, at 37°, and then samples of the culture were diluted appropriately and plated on streptomycin nutrient agar. After incubation at 37° for 48 hr., plates which had suitable numbers of well-isolated colonies were selected. The colonies on these plates were tested for bacteriocin production by the soft agar overlay method, using strain 1.9$\underline{str}^r$ as the indicator organism.

(2) Membrane filter method. The technique of mating bacteria on membrane filters can increase the efficiency of conjugation, presumably because the donor and recipient bacteria make a firmer contact than when mated in liquid media (Matney and Achenbach, 1962; Smith and Armour, 1966). It was therefore decided to use the membrane filter method in an attempt to detect transfer of bacteriocinogeny from strain 1.2 to a non-capsulate Klebsiella strain (KP105$\underline{str}^r$).

Donor and recipient bacteria were prepared and impinged on membrane filters as described by Matney and Achenbach (1962). The filters were incubated on nutrient agar plates at 37° for 16 hr., and then the bacteria were recovered by agitating the filters in sterile saline. Suitable dilutions of the resulting suspensions were plated on streptomycin nutrient agar, and the streptomycin resistant colonies which appeared were tested for bacteriocin production by the soft agar overlay method, using strain 1.9$\underline{str}^r$ as the indicator organism.

(3) High-frequency of transfer method. It has been shown that colicinogenic factor I can be transferred with very high frequency by cells of S. typhimurium that have just received

it, whereas cells carrying this factor are normally only poor donors (Ozeki and Howarth, 1961; Stocker, Smith and Ozeki, 1963). An experiment was therefore designed to screen for possible high-frequency transfer of the bacteriocinogenic factor from strain 1.2 to strain KP105$\underline{str}^r$.

Donor (1.2) and intermediate (KP105) bacteria, taken from nutrient agar plates, were grown in broth for 6 hr. at 37°. A 0.1 ml. aliquot of the donor culture was then mixed with 2 ml. of the intermediate culture (ratio 1:20); 1 ml. of the resulting mixture was added to 9 ml. of fresh broth and incubated overnight at 37°. Next morning, this culture was diluted 1/10 in fresh broth and incubated at 37° for a further 2 hr. A sample (0.5 ml.) of the final culture was then added to 4.5 ml. of a 22 hr. broth culture of the recipient strain KP105$\underline{str}^r$. The mixture was incubated at 37°, and at intervals of 2, 4 and 24 hr. samples were withdrawn, diluted appropriately, and plated on streptomycin nutrient agar. The colonies which appeared on this medium were replicated to streptomycin-free nutrient agar with sterile cocktail sticks, and tested for bacteriocin production by the soft agar overlay method, using strain 1.9 as the indicator organism.

Results

Over 250 $\underline{str}^r$ colonies were tested in each of the above experiments, but none were found to produce a bacteriocin active on the indicator strain 1.9 (or 1.9$\underline{str}^r$).

R factors - properties of K. aerogenes strain K66

When it was found that transfer of genetic material to Klebsiella strains could not be readily detected in any of the conjugation experiments described above, it was decided to examine systems of genetic transfer involving R factors. For this purpose, a Klebsiella strain (K66) carrying an R factor was obtained from Dr. N. Datta. This strain was originally isolated from a child suffering from paratyphoid, and the R factor which it carried conferred transmissible resistance to chloramphenicol, tetracycline, ampicillin and sulphonamides (N. Datta, personal communication).

Strain K66 formed large, convex, glistening, mucoid colonies on EMB-lactose, and examination in wet India ink films (Duguid, 1951) revealed that the cells were capsulate. In routine biochemical tests, strain K66 was found to behave in exactly the same way as K. aerogenes strain A3 and therefore appeared to be a typical K. aerogenes isolate. Phage sensitivity tests were then carried out with some 25 of the phages used routinely in this laboratory, and it was found that strains K66 and A3 belonged to the same phage type. Further relevant information was obtained by Dr. I.W. Sutherland (personal communication), who found that (1) the capsular polysaccharides of strains K66 and A3 are identical in chemotype, containing glucose, fucose, glucuronic acid and O-acetyl groups, (2) the lipopolysaccharides of strains K66 and A3 are identical in chemotype, containing galactose and glucosamine, and (3) the K66 and A3 capsular polysaccharides are sensitive

to a particular highly specific phage-induced fucosidase but insensitive to certain other phage-induced enzymes. The only differences which were detected were concerned with drug resistance characteristics - A3 was found to be sensitive to all four of the drugs to which K66 is resistant (i.e. chloramphenicol, tetracycline, ampicillin and sulphonamides).

From the above results, it appears that strains K66 and A3 are very similar and almost certainly belong to the same capsular serotype (type 54).

Choice of media for selection of drug resistant recipients

In transfer experiments, recipient cells which have acquired an R factor conferring resistance to certain drugs can be readily selected from a large population of R^- cells, simply by plating on a medium containing suitable concentrations of the appropriate drugs. However, unless the R factor is transferred at a very high frequency it is necessary to prevent the donor cells growing on the plates used for selection. Two methods were used to counterselect donor cells in this study: (1) high concentrations (1,000 µg./ml.) of streptomycin, to which donor cells were sensitive and recipient cells were resistant, were incorporated in the selective medium; (2) mixtures of auxotrophic donor cells and prototrophic recipient cells were plated on a minimal medium which could not support the growth of donor cells. Resistance characteristics controlled by R factors are rather rapidly expressed phenotypically (Watanabe, 1963a), and so it is not

usually necessary to incubate recipient cells for long periods prior to exposure to drugs.

Transfer of drug resistance to *K. aerogenes* strain A3*str*^{r}

In preliminary experiments, 0.02 ml. of a 6 hr. broth culture of the donor strain (K66, R^+) and 0.5 ml. of a 6 hr. broth culture of the recipient strain (A3*str*^{r}, R^-) were mixed in 5 ml. of fresh broth. The mixture was incubated overnight at 37°, and then diluted with 50 ml. of fresh, warm broth. After further incubation for 24 hr. at 37°, 0.2 ml. aliquots of the culture were withdrawn and plated on nutrient agar containing streptomycin and tetracycline (Str.Tc-NA). Cultures of the individual strains were plated on the same medium to provide controls for spontaneous mutants. Colonies were scored after incubation at 37° for 48 hr.

Results

It was found that colonies appeared on plates which had been spread with the mixed culture, but not on control plates. When several of the colonies from the mixture plates were replicated (with sterile cocktail sticks) to appropriate media, it was found that they were resistant to tetracycline, ampicillin, chloramphenicol and sulphonamides, whereas colonies picked from a plate culture of strain A3*str*^{r} were sensitive to these drugs. It seemed likely, therefore, that *K. aerogenes* strain K66 was capable of acting as a genetic donor, transferring multiple drug resistance to strain A3*str*^{r}.

Serial transfer of drug resistance between Klebsiella strains

Mixed cultures of the donor strain K66 and a recipient strain A3(0)*his*$^-$ *str*^{r} were prepared and treated as described in the previous experiment. Selection of drug resistant recipients was again accomplished by plating on Str.Tc-NA. Several of the colonies which appeared on mixture plates were picked and purified by serial subculture on the same medium. When the drug resistance characteristics of these isolates were tested by the disc method, it was found that all were resistant to chloramphenicol, ampicillin and sulphonamides as well as to tetracycline. It seemed likely, therefore, that the isolates were A3(0)*his*$^-$ *str*^{r} derivatives which had acquired the R factor from strain K66.

A further mixed culture was prepared as before, but this time the donor strain was a multiple drug resistant derivative of A3(0)*his*$^-$ *str*^{r}, and the recipient was A3(*str*^{s}). Selection of resistant recipients was accomplished by plating on tetracycline-minimal agar (Tc - min. A).

Results

Prototrophic tetracycline resistant colonies appeared on the Tc - min. A plates, and when such colonies were further tested they were found to be resistant to chloramphenicol, ampicillin and sulphonamides. The most likely explanation of this result seemed to be that an R factor carrying the four drug resistance markers had been transferred from K66 to A3(0)*his*$^-$ *str*^{r} in the first cross, and then from A3(0)*his*$^-$ *str*^{r} R$^+$ derivatives to A3*str*^{s} in the second cross. Support

for this suggestion was obtained when it was found that the *Tc*^{r} prototrophs resembled strain A3 in being capsulate and streptomycin sensitive, since for strain A3(0)*his*$^-$ *str*^{r} to acquire such a genotype by mutation it would be necessary to postulate three separate mutational events (*his*$^-$ to *his*$^+$, *str*^{r} to *str*^{s}, and non-capsulate to capsulate). It was therefore concluded that the *Tc*^{r} prototrophs represented A3 cells which had acquired multiple drug resistance as a result of infection with an R factor.

Studies on the mechanism of transfer of drug resistance between Klebsiella strains

It has been found that drug sensitive Klebsiella strains can acquire multiple drug resistance by incubation in mixed culture with strain K66. The bacteria which become resistant can then transfer multiple drug resistance to other strains. This transfer may or may not involve conjugation, and so an experiment was designed to determine whether drug resistance markers could be acquired from cell-free filtrates of strain K66.

An aliquot of an overnight broth culture of strain K66 was centrifuged and the supernatant was passed through a membrane filter (pore size 0.45 μ) to remove residual bacteria. A 10 ml. portion of the filtrate was inoculated with 1 ml. of an overnight culture of strain A3*str*^{r}, and the mixture was incubated at 37°. At 2 hr. intervals, the cells were removed by centrifugation and resuspended in fresh 10 ml. portions of

the K66 filtrate. After three such changes, the final suspension was incubated overnight at 37°. Samples of this culture were then spread on Str.Tc-NA plates.

To provide a positive control, a further 1 ml. aliquot of the original (unfiltered) K66 culture was mixed with 1 ml. of the recipient culture in 10 ml. of fresh broth. The resulting mixture was incubated at 37° overnight, and then samples were withdrawn and plated on Str.Tc-NA.

Results

Transfer of multiple drug resistance was detected in the control experiment, but no drug resistant recipients were recovered from the culture which had been treated with a cell-free filtrate of strain K66. This result suggested that intact donor cells were required for transfer, and since conjugation is the only known mechanism of genetic exchange in bacteria which involves direct contact between donor and recipient cells, it seemed likely that transfer of multiple drug resistance between the Klebsiella strains had occurred by R-mediated conjugation. Further support for this suggestion was obtained from a consideration of the other known mechanisms of genetic exchange.

In previous experiments, it was not found possible to transform strains of the A3 group with DNA extracted from donor cells, and great difficulty has been experienced in attempts to transform other members of the Enterobacteriaceae (Marmur et al., 1963). The negative results obtained with cell-free filtrates also suggested that transformation was not

involved in the transfer of multiple drug resistance between Klebsiella strains. The same results were used to argue that transfer did not occur by phage-mediated transduction, and it seemed even less likely that transduction was involved when it was found that culture filtrates of strain K66 did not contain a phage capable of producing zones of inhibition when plated on lawns of strain A3 (or indeed on lawns of any of the other Klebsiella strains in my collection). The converse finding that culture filtrates of strain A3 did not contain a phage active on strain K66 is also relevant, and has already been reported (see table 3.1).

Transfer of multiple drug resistance from K. aerogenes strain K66 to a range of recipient bacteria

A number of streptomycin resistant bacterial strains were obtained from other workers, and several more were isolated by plating mutagen-treated cultures on streptomycin nutrient agar as previously described. These strains were used as recipients in mixed culture experiments with K. aerogenes strain K66.

Donor and recipient bacteria were grown in broth at 37° for 6 hr. A 0.02 ml. aliquot of the donor culture (K66, R^+) was then mixed with 0.5 ml. of the recipient culture in 5 ml. of sterile broth, and the mixture was incubated at 37° overnight. Cultures of the individual strains were diluted and incubated in a similar manner to provide controls for spontaneous mutants. After incubation, 0.2 ml. samples of

Table 4.4

Recipient strain	No. of colonies/ml. on Str.Tc-NA
1.2*str*^{r}	0
1.9*str*^{r}	382
2-Park/*str*^{r}	184
A1*str*^{r}	171
A4*str*^{r}	38
418*str*^{r}	80
Styphimurium strain 907*str*^{r}	169
E. coli strain S23	1×10^6
E. coli strain S33	2×10^5

Transfer of multiple drug resistance from *K. aerogenes* strain K66 to a range of recipient bacteria

the mixed cultures and controls were withdrawn and spread on Str.Tc-NA plates to select drug resistant recipients.

Results

The results are presented in table 4.4. Colonies appearing on plates which had been spread with mixed cultures were thought to have acquired multiple drug resistance, and this was confirmed by replica plating with sterile velvet to media containing chloramphenicol and ampicillin. None of the control cultures were found to yield colonies on the Str.Tc-NA selection plates.

From the results, it can be seen that considerably fewer drug resistant recipients were recovered from mixed cultures of K66 and R^- Salmonella or Klebsiella strains than were recovered from K66 x E. coli K12 crosses. The significance of this finding is difficult to assess, however, because the mixed cultures were incubated at 37^o for 16 hr. prior to plating. During incubation for such a period, infected recipient cells would presumably replicate, thus increasing the absolute number of drug resistant bacteria in a culture, and the numbers of resistant cells recovered in the various crosses would therefore depend on the growth rates of the different strains. Newly infected cells might also transfer the R factor to other recipient bacteria, and indeed there is good evidence to suggest that recently infected bacteria transfer R factors much more readily than do cells in a culture which has carried the R factor through many generations (Watanabe, 1963b). Nevertheless, it was necessary to consider

results obtained with mating mixtures which had been incubated for relatively long periods, because it was found that very few or no drug resistant Klebsiella recipients could be detected in experiments where mating lasted for less than 12-16 hr.

Multiple drug resistance could be transferred to all of the recipients tested, with the exception of strain 1.2$\underline{str}^{r}$. The streptomycin resistant mutant of strain 1.2 which was used in this experiment was capsulate and formed particularly viscous colonies on normal media; it may be that the capsule was of an unusually impenetrable nature and was therefore capable of preventing direct contact between donor and recipient cells. However, other capsulate recipients (1.9, 2-Park, A1, A4 and 418) were found to acquire multiple drug resistance in mixed culture with strain K66, suggesting that genetic material can be transferred between capsulate bacteria quite readily although not at a very high frequency.

A further conclusion can be drawn from this experiment: it seems unlikely that the observed transfer of multiple drug resistance can be explained by phage-mediated transduction, since this would require a very unusual phage capable of infecting recipients of different genera and diverse surface properties. It was therefore assumed that $\underline{K.\ aerogenes}$ strain K66 normally transfers multiple drug resistance by conjugation.

Transfer of multiple drug resistance from K66 to capsulate and non-capsulate strains

In previous experiments, it was found that some 50 - 100/10^8 Klebsiella cells acquired an R factor as a result of incubation for 16 hr. in mixed culture with strain K66. When E. coli recipients were used, the proportion of cells which acquired the same R factor was very much greater. These results could indicate that only some of the cells in a Klebsiella culture were capable of acting as recipients, and so an experiment was designed to test whether the presence or absence of capsules would influence the proportion of recipient bacteria which acquired the R factor. The donor strain was K66, and the recipients were K. aerogenes strain A3str^r (capsulate) and K. aerogenes strain KP105str^r (non-capsulate).

Donor and recipient bacteria were grown in broth at 37° for 6 hr. A 0.02 ml. aliquot of the donor culture was then mixed with 0.5 ml. of a recipient culture in 5 ml. of sterile broth, and the mixture was incubated overnight at 37°. Cultures of the individual strains were diluted and incubated in a similar manner to provide controls for spontaneous mutants. After incubation, 0.2 ml. aliquots of the mixtures and controls were withdrawn and plated on Str.Tc-NA to select drug resistant recipients. The total number of viable recipient bacteria in each mixed culture was then determined by spreading measured aliquots of serial tenfold dilutions of the culture on streptomycin nutrient agar and counting the

colonies which appeared.

Results

Several experiments were carried out as described above, and in each case it was found that c. $50/10^8$ recipient cells acquired the R factor from strain K66. No evidence was found to suggest that the non-capsulate strain was capable of acquiring the R factor more efficiently than the capsulate strain.

Transfer of multiple drug resistance on membrane filters

The technique of mating bacteria on membrane filters can increase the efficiency of conjugation, presumably because the donor and recipient bacteria make a firmer contact than when mated in liquid media (Matney and Achenbach, 1962; Smith and Armour, 1966). It was therefore decided to use the membrane filter technique in attempts to increase the efficiency of mating between R^+ and R^- Klebsiella cells.

Donor (A3$\underline{str}^s R^+$) and recipient (A3(0)$\underline{his}^-\underline{str}^r R^-$) bacteria were prepared and impinged separately and together on membrane filters as previously described. Each filter was incubated on the surface of a nutrient agar plate at 37^o for 3 hr., and after this time the cells were recovered by vigorous shaking in saline. Measured aliquots of the saline suspension were then withdrawn and plated on (1) Str.Tc-NA, to determine transfer of the R factor, (2) Str-NA (after suitable dilution), to determine the total number of viable recipient cells present, and (3) Str.Tc-Min. A, to screen for transfer of the

presumably chromosomal *his* marker. Colonies were scored after incubation at 37° for 2-5 days. Str.Tc-NA and Str.Tc-Min.A plates which had been spread with control cultures of the individual strains were found to be barren.

Results

In a preliminary experiment, it was found that $73/10^8$ recipient bacteria had acquired multiple drug resistance within the 3 hr. which were allowed for mating on the filters. In later experiments, using the same technique and the same bacterial strains, as many as $120/10^8$ recipient bacteria became infected with the R factor within 3 hr. No transfer at all could be detected when equivalent numbers of cells of strains $A3R^+$ and A3(0)*his*$^-$ *str*^{r} were incubated in mixed broth culture for 3 hr., and so it appeared that mating on membrane filters was indeed more efficient than mating in broth. However, it should be noted that even the membrane filter technique allowed only a very small proportion of the recipient bacteria to become infected with the R factor.

In *E. coli* K12, R factors are thought to be capable of mediating chromosomal transfer, but this normally occurs at low rates - the frequencies of recombination for single genes are of the order of 10^{-8}/donor cell (Sugino and Hirota, 1962). It seemed unlikely, therefore, that it would be possible to detect chromosomal transfer in the above experiments, since at the most only $120/10^8$ recipient bacteria became infected with the R factor. In fact, no colonies appeared on the Str.Tc-Min.A plates and so no *his*$^+$ recombinants were detected.

It was also found that none of the colonies which had appeared on the Str.Tc-NA plates (i.e. R^+ recipients) were able to grow when replicated to histidine-free media. Chromosomal transfer could not therefore be reported.

Further experiments were carried out in exactly the same way, using strain K66 as the donor and strains A3*pro*$^-$*str*^{r}, KP105/S1.*str*^{r}, and *E. coli* S33 as recipients, but again transfer of chromosomal material was not detected.

Transfer of multiple drug resistance in HFRT systems

A number of attempts were made to prepare high-frequency resistance transfer (HFRT) systems involving Klebsiella strains. The principle of the technique has been discussed in the introduction to this section, and the actual method used was based on one described by Datta, Lawn and Meynell (1966).

Donor (R^+) and intermediate (R^-) bacteria, taken from nutrient agar plates, were grown in broth for 6 hr. at 37°. A 0.2 ml. aliquot of the R^+ culture was then mixed with 10 ml. of the R^- culture (ratio 1:50); 1 ml. of the resulting mixture was added to 9 ml. of fresh broth and incubated at 37° overnight. Next morning, this culture was diluted 1/20 in fresh broth and incubated at 37° for a further 2 hr. Crosses were then carried out by mixing 0.5 ml. of the final donor/intermediate culture with 4.5 ml. of a 22 hr. broth culture of the recipient bacteria. After incubation at 37° for 40 min. (or in some cases for a prolonged period) to allow mating, measured

aliquots were withdrawn from the mixture and plated on (1) Str.Tc-NA, to select drug resistant recipients, and (2) Str-NA (after suitable dilution), to determine the total number of viable bacteria present. Samples of the donor/intermediate mixture and of the recipient culture were also plated on Str.Tc-NA, to provide controls for spontaneous mutants.

Results

The results are presented in table 4.5. When E. coli strain S33 was used as a recipient, it was found that transfer of the R factor occurred within 40 min., even when no intermediate strain was used. However, the proportion of recipient cells that became infected was not increased by prior incubation of strain K66 with drug sensitive (R^-) Klebsiella strains, and in fact it was found that the proportion of infected recipients was lower when Klebsiella intermediates had been used. This result probably reflects the presence of fewer donor cells in the donor/intermediate mixtures than in a pure K66 culture. Transfer of drug resistance to E. coli strain S33 was increased more than tenfold by prior incubation of the donor strain (K66) with another E. coli strain (703), and presumably most of the competent donors in the K66/703 mixture were cells of strain 703 which had recently received the R factor from K66. A further conclusion which can be drawn from this group of results is that strains KP105 and A3 are probably ineffective as intermediates because they are not capable of acting as efficient donors even although they may have recently acquired the R factor.

Table 4.5

Donor	Intermediate	Recipient	No. of cells that acquired resistance/10^8 recipient cells
K66	-	S33	140
K66	A3	S33	76
K66	KP105	S33	60
K66	703	S33	1600
K66	703	A3<u>str</u>^{r}	0
K66	703	KP105<u>str</u>^{r}	0
K66	703	*KP105<u>str</u>^{r}	40
K66	A3	A3<u>str</u>^{r}	0

* Recipient culture had been diluted 1/20 and incubated at 37^o for 2 hr. before use.

Transfer of multiple drug resistance in HFRT systems

When Klebsiella strains were used as recipients, a rather different situation became apparent. Firstly, no transfer of multiple drug resistance was detected in crosses between a K66/A3 donor mixture and an A3<u>str</u>^{r} recipient culture, suggesting that cells of strain A3<u>str</u>^{r} were not capable of acting as efficient recipients. Secondly, the K66/703 mixture which transferred drug resistance relatively efficiently to <u>E. coli</u> strain S33 did not transfer drug resistance to 22 hr. broth cultures of Klebsiella strains A3<u>str</u>^{r} and KP105<u>str</u>^{r} in 40 min. mating experiments. This result confirmed the view that the Klebsiella strains were poor recipients, since the K66/703 mixture had already been shown to contain a fairly high proportion of competent donor cells. In prolonged mating experiments, it was found that transfer of drug resistance from a K66/703 mixture to strain KP105<u>str</u>^{r} could be detected within 1-3 hr., but the proportion of recipient bacteria which acquired the R factor was low (of the order of $4/10^8$ recipient cells), and even after 22 hr. only $2 \times 10^3/10^8$ recipient cells had become drug resistant.

In a further experiment, an overnight broth culture of strain KP105<u>str</u>^{r} was diluted 1/20 in fresh broth and incubated at 37^o for 2 hr. The bacteria in the resulting culture were then used as recipients in a cross with a K66 x 703 donor/intermediate mixture. From the results shown in table 4.5, it can be seen that multiple drug resistance could be transferred to recipient bacteria prepared in this way within 40 min. of mixing with the donor culture. However, only some $40/10^8$

recipient cells were found to acquire the R factor within this period, and attempts to obtain higher levels of transfer in repeat experiments were unsuccessful.

Properties of E. coli K12 strain J5 and the R factor which it carries

It has been shown that R factors are capable of mediating conjugation, but in most bacteria carrying R factors conjugating ability is absent, which implies repression by regulatory genes (Datta, Lawn and Meynell, 1966; Meynell and Datta, 1967). Thus if wild-type R^+ cultures conjugate only rarely because of repression, mutant R factors lacking the repressor should occur. Such mutants of fi^+ R factors have recently been isolated (Meynell and Datta, 1967), and the R factor carried by strain $J5R^+_{1-14}$ is one of them (N. Datta, personal communication). This factor confers resistance to ampicillin, streptomycin, chloramphenicol and sulphonamides, and can be transferred between E. coli K12 strains at high frequency.

It was decided to examine the behaviour of the R_{1-14} factor in Klebsiella cells, and it was therefore necessary to consider the choice of a selective medium for use in transfer experiments. As before, recipient cells which had acquired the R factor could be readily selected by plating on media containing drugs to which the R factor confers resistance, but since earlier experiments had suggested that Klebsiella cells were poor recipients, it seemed likely that it would again be necessary to prevent donor cells growing on the plates used

for selection. Now, $J5R^{+}_{1-14}$ is a *lac*$^{+}$ *pro*$^{-}$ *met*$^{-}$ derivative of *E. coli* K12, and growth of this strain can therefore be prevented by plating on a minimal medium lacking methionine and proline. However, in preliminary experiments it was found that the drug sensitive prospective recipient strains (*K. aerogenes* A3 and derivatives of this strain) were capable of forming colonies on a minimal medium containing normal concentrations of the appropriate drugs (ampicillin and chloramphenicol). Tests with minimal media containing higher concentrations of these drugs proved unsuccessful, because it was found that (1) no drug resistant colonies were recovered when R^{+}_{1-14} x R^{-} mating mixtures were plated on such media, and (2) cultures of strain $A3R^{+}_{1-14}$ (i.e. cultures which had acquired resistance to ampicillin and chloramphenicol as a result of infection with the R_{1-14} factor in other experiments) were incapable of growing on such media. It would almost certainly have been possible to obtain satisfactory results with intermediate concentrations of drugs.

Another method which was used to counterselect donor bacteria in previous experiments involved plating mixtures of *str*^{s} donors and *str*^{r} recipients on media containing high concentrations of streptomycin (1,000 µg./ml.). Now, the R_{1-14} factor confers transmissible resistance to streptomycin, and it might therefore be thought that this method would be unsuitable for use in R^{+}_{1-14} x R^{-} crosses. However, it has been found that certain R factors confer resistance to relatively low levels of streptomycin (of the order of 100 µg./ml. or less:

Datta, 1965), and since Klebsiella recipients resistant to 1,000 µg./ml. (or more) of streptomycin can be readily isolated, it seemed likely that it would in fact be possible to prevent growth of an R^+_{1-14} donor strain simply by plating on media containing the higher level of streptomycin. Preliminary tests showed that strain $J5R^+_{1-14}$ could not grow on nutrient agar containing 1,000 ug./ml. of streptomycin, and so it was decided to use the streptomycin counterselection method in transfer experiments.

Transfer of the R_{1-14} factor to a Klebsiella recipient

Donor ($J5R^+_{1-14}$) and recipient (A3$\underline{str}^r$) bacteria were prepared and impinged separately and together on membrane filters as previously described. The filters were incubated on nutrient agar plates at 37° for 2 hr. to allow mating, and then the bacteria were recovered by agitating the filters in saline. Samples of the resulting suspensions were withdrawn and plated on nutrient agar containing streptomycin, chloramphenicol and ampicillin (Str.Cp.Ap-NA) to select drug resistant recipients. Colonies were scored after incubation at 37° for 48 hr.

Results

No colonies appeared on plates which had been spread with control suspensions of the individual strains, whereas several colonies appeared on plates which had been spread with the mating mixture. A number of these colonies were picked and purified by re-streaking on the medium used for selection.

Tests for drug sensitivity were then carried out by the disc method, and it was found that recipients which had acquired resistance to ampicillin and chloramphenicol were also resistant to sulphonamides. No measurement was made of the total number of recipients plated in this experiment, but it was estimated that roughly $100/10^8$ recipient bacteria had acquired multiple drug resistance.

Transfer of the R_{1-14} factor from strain J5 to a non-capsulate derivative of strain A3

It has been shown that strain $J5R^+_{1-14}$ is an efficient donor of the R_{1-14} factor, and transfer of this factor to an E. coli K12 recipient occurs readily in broth (Meynell and Datta, 1967; N. Datta, personal communication). In the previous experiment, it was found that the R_{1-14} factor could be transferred to a Klebsiella recipient on a membrane filter, and it was therefore decided to test for transfer in broth. The recipient strain used for this purpose was KP105str^r, a non-capsulate mutant of K. aerogenes A3. This strain was chosen because it had previously been found that KP105str^r cells in a 2 hr. broth culture could acquire an R factor from an HFRT Klebsiella/E. coli mixture within 40 min.

In a preliminary experiment, overnight broth cultures of the donor ($J5R^+_{1-14}$) and the recipient (KP105str^r) were diluted 1/20 in fresh broth and incubated at 37^o for a further 2 hr. A 0.5 ml. aliquot of the donor culture was then mixed with 4.5 ml. of the recipient culture, and the mixture was

incubated at 37° for 40 min. After this time, 0.2 ml. samples were withdrawn and plated on Str.Cp.Ap-NA. No drug resistant recipients were detected by this method, and so it was decided to test for transfer of the R_{1-14} factor to strain KP105<u>str</u>^{r} in a prolonged mixed culture experiment.

Donor (J5R^{+}_{1-14}) and recipient (KP105<u>str</u>^{r}) bacteria, taken from nutrient agar plates, were grown in broth for 6 hr. at 37°. A 0.02 ml. aliquot of the donor culture was then mixed with 0.5 ml. of the recipient culture in 5 ml. of sterile broth, and the mixture was incubated at 37° for 16 hr. Cultures of the individual strains were diluted and incubated in a similar manner to provide controls for spontaneous mutants. After incubation, samples were withdrawn from the mixture and controls, diluted if necessary, and spread on Str.Cp.Ap-NA plates to select drug resistant recipients. A further sample of the mixture was diluted appropriately in saline, and then 0.1 ml. aliquots of the dilutions were spread on Str-NA to provide a measure of the total number of viable recipients present. Colonies were scored after incubation at 37° for 48 hr.

<u>Results</u>

It was found that many colonies appeared on Str.Cp.Ap-NA plates which had been spread with the mixed culture, but none appeared on control plates. Several of the colonies from the mixture plates were picked and purified by re-streaking on fresh Str.Cp.Ap-NA plates, and when the resulting sub-lines were tested for drug sensitivity by the disc method it was

found that all of them had acquired resistance to sulphonamides as well as to ampicillin and chloramphenicol. Morphologically, the drug resistant colonies resembled strain KP105*str*^{r} and differed from strain J5R$^+_{1-14}$. It seemed likely, therefore, that the colonies which appeared on the Str.Cp.Ap-NA plates were KP105*str*^{r} derivatives which had acquired the R_{1-14} factor.

From the numbers of colonies appearing on the Str.Cp.Ap-NA and Str-NA plates, it was estimated that approximately $10^4/10^8$ recipient cells had acquired the R_{1-14} factor after incubation for 16 hr. with strain J5R$^+_{1-14}$.

Transfer of the R_{1-14} factor from strain J5 to *K. aerogenes* strain W107

During the course of this work, a spontaneous mutant of *K. aerogenes* strain A3(0)*his*$^-$*str*^{r} was observed on an EMB-lactose plate. The mutant was designated W107, and it differed from the parent organism in forming slightly flatter, less smooth, colonies on EMB-lactose agar. In preliminary experiments, it was found that a high proportion of W107 cells (more than $5 \times 10^4/10^8$ recipients) became drug resistant after overnight growth in mixed culture with J5R$^+_{1-14}$. This result suggested that it might be possible to transfer drug resistance to strain W107 in a 40 min. mating experiment.

An overnight broth culture of strain J5R$^+_{1-14}$ was diluted 1/20 in fresh broth and incubated at 37^o for a further 2 hr. A 0.5 ml. aliquot of this donor preparation was then mixed with 4.5 ml. of an overnight broth culture of strain W107.

Table 4.6

	No. of colonies/ml. plated on Str.Cp-NA	No. of colonies/ml. plated on Str-NA
$J5R^{+}_{1-14}$ control	0	-
W107 (*his*$^{-}$ *str*^{r}R^{-}) control	540	-
$J5R^{+}_{1-14}$ x W107 mixture	2600	1×10^{9}

Transfer of the R_{1-14} factor from strain J5 to *K. aerogenes* strain W107 in a 40 min. mating experiment

Control cultures of the individual strains were also prepared. After incubation for 40 min. at 37°, samples were withdrawn from the mixture and controls and plated on Str.Cp-NA to select drug resistant recipients. A further sample of the mixture was diluted appropriately and plated on Str-NA, to provide a measure of the total number of viable recipient cells present.

Results

The results are presented in table 4.6. It can be seen that a number of colonies were found on Str.Cp-NA plates which had been spread with the recipient strain (W107) alone, but tests by the disc method showed that none of these colonies contained bacteria which were resistant to ampicillin or sulphonamides. It seemed likely, therefore, that such colonies represented spontaneous chloramphenical resistant (Cp^r) mutants. A number of colonies were also found on Str.Cp-NA plates which had been spread with the mixed culture. Forty of these colonies were picked, purified by re-streaking on the medium used for selection, and then tested for drug resistance by the disc method. It was found that 7/40 of the colonies were resistant to chloramphenicol, but sensitive to ampicillin and sulphonamides, and it was presumed that these represented spontaneous Cp^r mutants. The remaining 33/40 were resistant to chloramphenicol, ampicillin and sulphonamides, and these were presumed to be W107 derivatives which had acquired the R_{1-14} factor from strain $J5R^+_{1-14}$. The problem of background growth of Cp^r mutants could have been overcome

by the addition of ampicillin to the selective medium, but powdered ampicillin was not available when this experiment was carried out.

After making suitable corrections for the spontaneous mutations of the recipient strain, it was calculated that approximately $200/10^8$ recipient cells had acquired multiple drug resistance within the 40 min. mating period. This value may be rather low, since it is probable that the mating mixture contained a further number of recipient cells which had acquired the R factor, but which had not completely expressed resistance before plating on the chloramphenicol-containing medium. Evidence obtained with substrains of E. coli K12 suggests that phenotypic expression of chloramphenicol resistance takes about 10 min. after infection by R factors (Watanabe, 1963a), and so recipient cells which had acquired the R_{1-14} factor late in the 40 min. incubation period may not have been detected on the Str.Cp-NA selection plates.

In a further mating experiment, a drug resistant (R^+_{1-14}) derivative of strain W107 was incubated overnight at 37° in mixed culture with strain KP105str^s. Since the donor strain (W107) required histidine, the medium used for selection of drug resistant recipients was Cp-Min.A agar. As previously mentioned, this medium does not completely prevent growth of recipient Klebsiella strains, and indeed a high degree of background growth was observed when samples of the W107 x KP105str^s mixture were plated on Cp-Min.A and incubated at 37°

for 24 hr. No attempt was made to obtain quantitative results, but when several colonies had been picked and purified by re-streaking on Cp-Min.A it was found possible to identify among them a number of multiple drug resistant derivatives of the recipient, KP105*str*^{s}. It was concluded from this finding that the drug resistant derivative of strain W107 was capable of transferring multiple drug resistance, and had therefore acquired the R_{1-14} factor in the first mating experiment described above.

Attempts to detect R mediated chromosomal transfer

In this experiment, attempts were made to detect transfer of a chromosomal marker from a drug resistant derivative of the prototrophic strain KP105*str*^{s} to W107, a non-capsulate, *his*$^{-}$*str*^{r} derivative of strain A3.

An overnight broth culture of the donor strain (KP105*str*^{s} R^{+}_{1-14}) was diluted 1/20 in fresh broth and incubated at 37^{o} for a further 2 hr. A 0.5 ml. aliquot of the resulting culture was then mixed with 4.5 ml. of an overnight broth culture of the recipient strain (W107, *his*$^{-}$*str*^{r}R^{-}). Control cultures of the individual strains were also prepared. After incubation at 37^{o} for 40 min. to allow mating, 0.1 ml. samples of the mixed culture and controls were withdrawn and plated on (1) Str-min.A, to select prototrophic recombinants, (2) Str.Cp-min.A, to select chloramphenicol resistant prototrophic recombinants, and (3) Str.Cp-NA, to select R^{+} recipients. Colonies were scored after incubation at 37^{o} for 48 hr.

Results

As in the previous experiment, several colonies were found on Str.Cp-NA plates which had been spread with the recipient strain (W107) alone, and it was again presumed that these were spontaneous $\underline{Cp}^r$ mutants. However, the number of colonies appearing on Str.Cp-NA plates which had been spread with the mixed culture was not significantly greater than the number on W107 control plates. Moreover, when colonies from the Str.Cp-NA mixture plates were replicated with sterile cocktail sticks to minimal A containing sulphonamide, it was found that none of them were capable of growing. It seemed likely, therefore, that transfer of the R_{1-14} factor to strain W107 had not occurred, and so it was not surprising that no more prototrophs were recovered on Str-min.A or Str.Cp-min.A mixture plates than were recovered on control plates.

In a further experiment, donor (KP105$\underline{str}^s$ R^+_{1-14}) and recipient (W107 R^-) bacteria were grown on broth at 37° for 6 hr. A 0.02 ml. aliquot of the donor culture was then mixed with 0.5 ml. of the recipient culture in 5 ml. of sterile broth, and the mixture was incubated at 37° overnight. Cultures of the individual strains were diluted and incubated in a similar manner to provide controls for spontaneous mutants. After incubation, 0.1 ml. samples were withdrawn from the mixture and controls, diluted if necessary, and plated on Str-min.A, Str.Cp-min.A, and Str.Cp-NA as before. Transfer of the R_{1-14} factor was observed when the bacteria were mated in this way, but again no more prototrophic

colonies were recovered on Str-min.A or Str.Cp-min.A mixture plates than were recovered on control plates, i.e. transfer of the (presumably) chromosomal his marker to strain W107 was not observed.

Sensitivity of bacteria carrying the R_{1-14} factor to male-specific bacteriophage

In previous experiments, it has been found possible to transfer multiple drug resistance from E. coli strain $J5R^{+}_{1-14}$ to Klebsiella recipients. The recipient bacteria presumably acquired drug resistance as a result of infection with the R_{1-14} factor, and should therefore contain this factor. Now, R_{1-14} is a mutant R factor in which conjugating ability is not repressed, and E. coli K12 cells harbouring R_{1-14} become sensitive to the male-specific phage MS-2 (Datta, personal communication). It was therefore of interest to determine whether Klebsiella cells harbouring R_{1-14} would also become sensitive to MS-2.

Lysates of phage MS-2 containing approximately 2×10^{10} p.f.p./ml. were prepared by the soft agar layer method (Adams, 1959), the host bacterial strain being $J5R^{+}_{1-14}$. Serial ten-fold dilutions of the lysate were prepared, and then three bacterial strains ($J5R^{+}_{1-14}$, $A3str^{r}R^{+}_{1-14}$, and $KP105R^{+}_{1-14}$) were tested for sensitivity to the phage. This was done by placing drops of each dilution of the lysate on soft agar overlays seeded with the appropriate bacterial strain. Duplicate test plates were prepared; one set was incubated at 37^{o}, and

the other set was incubated at 42° because Dettori, Maccacaro and Turri (1963) have shown that sensitive cells are more efficiently lysed by F specific phages at this temperature.

Results

The male-specific phage MS-2 was found to produce plaques on strain $J5R^{+}_{1-14}$ whether the plates were incubated at 37° or 42°, but no plaques were observed in lawns of the R^{+} Klebsiella strains at either temperature. Nevertheless, it is possible that a low proportion of the cells in the Klebsiella cultures were sensitive to MS-2. Under such circumstances plaque formation would probably not be observed, since plaques only become visible when a relatively high proportion (c. 50%) of the bacteria are sensitive and can therefore be lysed (Meynell and Datta, 1966b).

Adsorption of phage MS-2 by bacteria carrying the R_{1-14} factor

It has been found that 70-90% of the cells in a culture of strain J5 produce specialised (F) pili when infected with the R_{1-14} factor (Datta, personal communication), and it appears that these pili are the receptors for phage MS-2 (Datta *et al.*, 1966). The sensitivity of $J5R^{+}_{1-14}$ to phage MS-2 can be readily tested by plaque formation on seeded agar lawns, but Klebsiella strains ($A3\underline{str}^{r}R^{+}_{1-14}$ and $KP105R^{+}_{1-14}$) which carry the R_{1-14} factor do not show MS-2sensitivity when tested in this way. The failure to detect plaque formation with MS-2 may be due to inability of the Klebsiella cells to support growth of the phage. Alternatively, MS-2 may

resemble several other phages which are capable of entering and leaving certain bacterial cells without causing lysis (Hofschneider and Preuss, 1963; Hoffmann-Berling and Maze, 1964). Thus a high proportion of the cells in cultures of strains KP105R^+_{1-14} and A3$\underline{str}^r R^+_{1-14}$ may synthesise F pili without necessarily showing plaque formation. If this is the case, it should be possible to demonstrate adsorption of MS-2 particles by R^+_{1-14} Klebsiella strains.

Three bacterial strains (J5R^+_{1-14}, KP105$\underline{str}^r R^+_{1-14}$, and KP105$\underline{str}^r R^-$) were tested for ability to adsorb phage MS-2. Overnight TYeCa broth cultures of the strains were prepared, and 9.5 ml. aliquots were mixed with 0.5 ml. aliquots of an MS-2 lysate. The multiplicity of infection was about 0.1, since the mixtures contained approximately 2×10^9 bacteria/ml. and 2×10^8 p.f.p. of phage MS-2 per ml. The mixtures were incubated at 37^o for 10 min. to allow phage adsorption, and then centrifuged in the cold at 8,000 g for 10 min. to deposit the bacteria plus adsorbed phage particles. The supernatants were passed through membrane filters to remove residual bacteria, and assayed for phage by the soft agar layer method (Adams, 1959) with strain J5R^+_{1-14} as the indicator organism. Plaques were scored after incubation at 42^o for 16 hr.

Results

From the results (table 4.7), it can be seen that cells of strain J5R^+_{1-14} adsorbed approximately 75% of the plaque-forming particles. On the other hand, cells of strain KP105$\underline{str}^r R^+_{1-14}$ adsorbed only 25%, and since cells of strain

Table 4.7

	Input of phage p.f.p./ml.	Titre of supernatant p.f.p./ml.	% adsorbed
$J5R^{+}_{1-14}$	2×10^{8}	5×10^{7}	75
KP105<u>str</u>$^{r}R^{+}_{1-14}$	2×10^{8}	1.5×10^{8}	25
KP105<u>str</u>$^{t}R^{-}$	2×10^{8}	1.6×10^{8}	20

Adsorption of phage MS-2 by various recipient strains

KP105str^{r}R$^-$ adsorbed 20%, it did not seem likely that the adsorption by KP105str^{r}R$^+_{1-14}$ was significant. The experiment was repeated twice more, but the results were of the same order as those reported in table 4.7.

Incubation of Klebsiella cells harbouring the R_{1-14} factor with phage MS-2

In an attempt to demonstrate the growth of phage MS-2 in R^+_{1-14} Klebsiella cells, an experiment was carried out using a technique described by Horiuchi and Adelberg (1965).

A 6 ml. aliquot of TYeCa broth was inoculated with 0.2 ml. of an overnight broth culture of strain KP105str^{r}R$^+_{1-14}$, and with 0.2 ml. of a lysate of phage MS-2 containing c. 6 x 10^5 p.f.p./ml. A sample was immediately withdrawn from the mixture, passed through a membrane filter to remove bacteria, and assayed for phage by the soft agar layer method (Adams, 1959). The culture was then incubated at 37^o for 12 hr., and after this time a further phage assay was carried out. Plaques were scored after incubation at 42^o for 16 hr.

Results

No increase in plaque-forming titre was observed when phage MS-2 was incubated with K. aerogenes strain KP105str^r R$^+_{1-14}$. The burst size of phage MS-2 is normally large (2-3,000 in E. coli or Proteus mirabilis: Horiuchi and Adelberg, 1965), and so even if only a small proportion of the cells in the KP105str^{r}R$^+_{1-14}$ were phage-sensitive it should have been possible to detect an increase in titre in the

above experiment. It was therefore concluded that cells of strain KP105<u>str</u>^{r} which harboured the R_{1-14} factor did not become sensitive to the male-specific phage MS-2.

DISCUSSION

In attempting to establish a new system of genetic analysis for a group of bacteria, the first problem which has to be faced is that there will almost certainly be very little information available about previous attempts with the same group, and yet there will be a plethora of information about studies with other, possibly closely related, organisms. When this study was initiated, there were reports of unsuccessful attempts to detect genetic exchange in Klebsiella by Sherman and Wing (1937), Balows et al. (1955) and Clarke (1960, 1961), and a number of other pieces of peripheral information which emerged as incidental observations in studies oriented towards other organisms. On the other hand, studies with E. coli K12, and to a lesser extent with Salmonella, Shigella and Proteus, have led to a literature which can only be described as vast. Nor is it possible to restrict consideration to studies with organisms which are thought to be close relatives of the group in question, because the type of genetic interaction characteristic of a particular organism may not correlate with present conceptions of its taxonomic status.

It is not easy to suggest explanations for the various unsuccessful attempts to detect transformation of enteric bacteria mentioned in the introduction to section II, and the negative results obtained in the present study are equally difficult to assess. The first question which arises concerns the suitability of the DNA preparations used. This is a variable which can only be adequately tested in a system

where positive results are obtained, but it can be said that most of the techniques used for DNA extraction were techniques which had previously been applied successfully to transformation of other bacterial groups. In addition, certain of the extraction procedures (e.g. penicillin lysis of normal cells: Balassa, 1963) were extremely mild, and should not have led to degradation of potentially transforming fragments of high molecular weight DNA.

It is possible, however, that cultures of the recipient strains contained an extracellular DNase which was capable of depolymerising homologous DNA; genetically active DNA would therefore have been destroyed before coming into contact with the cells. No attempt was made to test organisms of the K. aerogenes A3 group for production of such an enzyme, but Rothberg and Swartz (1965) tested a large number of Klebsiella (Aerobacter) strains and found that none of them were active in this respect.

A more likely explanation would seem to be that DNA fragments potentially capable of causing transformation were unable to enter recipient cells. For example, organisms of the Klebsiella group (and possibly all enteric bacteria) may lack the enzymatically active receptor sites which have been associated with uptake of transforming DNA in Streptococcus and Diplococcus (Pakula, 1965; Tomasz and Mosser, 1966). Alternatively, the receptors may be present, but may be located deep in the bacterial membrane complex. Access to the sites may therefore be restricted by the more superficial

cell wall material, and although it is conceivable that treatment with penicillin facilitates access in E. coli (e.g. in the systems described by Chargaff et al., 1957, and Wacker and Laschet, 1961) the same may not be true of Klebsiella.

However, it is by no means certain that there is an absolute requirement for enzymatically active receptor sites in transformation systems. A bacterium which is capable of taking up genetically active DNA may merely have an increased permeability to large molecules resulting from discontinuities in the cell wall, in which case it is possible that the attempts to detect transformation of Klebsiella strains were unsuccessful because the various treatments to which the cells were subjected did not yield suitably permeable recipients. Moreover, the physiological factors which determine competence are known to vary markedly from species to species (Schaeffer, 1964), and it may be that Klebsiella cells will prove to be susceptible to transformation in a particular but as yet undiscovered environment.

Assuming that DNA fragments potentially capable of causing transformation were able to enter Klebsiella cells, there are at least two other mechanisms which could conceivably prevent the recovery of transformants. Firstly, exogenous DNA may be depolymerised by an intracellular DNase, even although the host cell's own DNA would presumably be unaffected by such an enzyme. This difficulty should not have arisen in experiments where (as in most cases) the donor and recipient bacteria were derivatives of a single strain and the exogenous DNA was

presumably therefore virtually identical with the DNA of the recipient cells. However, the process of extraction may have so altered the DNA - possibly simply by reducing the size of the molecular fragments - that it became susceptible to depolymerisation by an intracellular DNase.

A second intracellular mechanism which might prevent the detection of transformants is suggested by the finding of Clark and Margulies (1965) that certain mutants of E. coli K12 are unable to participate in recombination (recombination-deficient or rec⁻ mutants). It is conceivable that some Klebsiella strains, and in particular the derivatives of K. aerogenes A3, are incapable of recombining with exogenous DNA, possibly because they lack an enzyme which is necessary for the recombination process to occur. Support for this hypothesis can only be tentative, but the finding that derivatives of strain A3 can accept and retain non-chromosomal genetic determinants (e.g. F-lac, R factors) is not contradictory since rec⁻ mutants of E. coli K12 also behave in this way. In this context, it would be unreasonable to consider the failure to detect chromosomal recombinants in transduction and conjugation experiments with strain A3 as corroborative evidence, because it is not known whether any of the phages tested were actually capable of mediating transduction, and it seems likely that mating pair formation did not occur at sufficiently high levels to allow conjugal transfer to be detected. In any case, Signer and Beckwith (1966) have recently presented evidence to show that certain phages are

themselves capable of producing the enzyme which is necessary for recombination to occur. The possibility that certain Klebsiella strains are incapable of participating in recombination should therefore be borne in mind until transformation or conjugation systems involving chromosomal transfer are reported.

In experiments designed to screen for transduction in Klebsiella the first significant results were obtained when pairs of auxotrophic mutants were incubated together on the surface of minimal agar plates. Attempts to characterise the mechanism of the genetic exchange led to the following conclusions: (1) conjugation was not involved, because cell-free filtrates were active in transfer, (2) transformation was not involved, because the genetic activity of a culture supernatant was not inhibited by treatment with DNase, and (3) phage-mediated transduction was probably involved, because there was an association between plaque-forming titre and transducing ability in high speed centrifugation experiments.

That the system was not one of specialised transduction emerges from the following points: (1) phage released by lysis of sensitive bacteria was apparently active in genetic transfer, whereas phages competent in specialised transduction are only found in lysates from lysogenic bacteria (Hartman, 1963), and (2) the phage was apparently capable of transducing a wide range of markers (including amino acid, vitamin, purine, pyrimidine, sugar utilisation and drug resistance markers), whereas phages competent in ~~general~~ specialised transduction are usually capable of transducing only those markers which are situated close to a

small number of preferred locations on the chromosome.

It is conceivable that the genetically active fraction in the cell-free filtrates was not phage, but was associated with phage in sedimentation and in yield during lysis of a donor strain. Thus in order to confirm that the genetic vector was in fact bacteriophage it would be necessary to test the ability of specific antiphage antiserum to abolish the genetic activity from a cell-free filtrate. However, in all transduction experiments with phage PW52 it was found that lysates which had a low plaque-forming titre had a low transducing ability (and vice versa), and so it seems highly likely that phage PW52 is capable of mediating generalised low frequency transduction.

One of the aims of the present study was to attempt to establish a system which would allow genetic analysis of capsulation phenomena in the Klebsiella group, and when it was found that genetic information could be exchanged between capsulate strains in a process which was thought to be phage-mediated transduction, it seemed likely that a suitable system had been found. However, preliminary tests showed that treatment of non-mucoid mutants of strains W52 and W70 with phage PW52 grown on a mucoid host did not lead to the recovery of mucoid transductants, and indeed the various non-mucoid mutants which were further tested did not yield streptomycin resistant or prototrophic transductants either. A possible explanation for this behaviour, based on the observation that phage PW52 was found to have a reduced efficiency of plating

when titrated on a non-mucoid host, has already been suggested. This explanation depends upon the untested hypothesis that all of the non-mucoid mutants which were used in this study and which should normally have been sensitive to phage PW52 (i.e. derivatives of strain W70) would show a reduced efficiency of plating with phage PW52. It is also necessary to postulate that the reduced efficiency of plating observed with derivatives of strain W70 would be reflected in failure of non-mucoid derivatives of the lysogenic, immune strain W52 to acquire genetically active DNA from a high proportion of the phage particles in a PW52 lysate. This cannot be tested directly, but it might be possible to obtain non-lysogenic ("cured") derivatives of non-mucoid mutants of strain W52 by treatment of cultures with ultraviolet light (Zinder, 1958), and then to determine the efficiency of plating of phage PW52 on the resulting phage-sensitive bacteria. If it is found that phage PW52 shows a reduced efficiency of plating on such derivatives, then it would seem likely that only a proportion of the phage particles in a PW52 lysate are capable of injecting their DNA into non-mucoid recipients, whether the recipients are lysogenic and immune or not. This may reflect poor adsorption of phage particles by the non-mucoid cells, possibly because the capsular polysaccharide itself is involved in phage adsorption, or may indicate that the lysates used in this study contained two types of phage, one type being capable of infecting mucoid (capsulate) bacteria only, and the other type being capable of infecting both mucoid and

non-mucoid alike.

In order to test the above theories it would be necessary to isolate a large number of non-mucoid derivatives, and it would be better to use the PW52-sensitive strain W70 since the efficiency of plating of phage PW52 on each mutant could then be determined directly. If any of the mutants were found to have an efficiency of plating approaching the value which is typical of the capsulate host, it would then be possible to test lysogenised derivatives for ability to be transduced by phage PW52. On the other hand, if none of the mutants were found to be transducible or to have a high efficiency of plating, then it would be necessary to consider another approach. One possibility is that phage PW52 grown to high titre on a non-mucoid host could be used in attempts to transduce capsulate recipients. Preliminary experiments were in fact carried out to this end, but phage preparations with sufficiently high titres could not be obtained. A further possible approach would involve attempts to isolate a mutant of phage PW52 which was capable of infecting cells of mucoid and non-mucoid strains with equally high efficiency.

The capsular and slime polysaccharides produced by K. aerogenes strain W70 both contain galactose, mannose and glucuronic acid, and the lipopolysaccharide of strain W93 (a non-capsulate mutant of strain W70) contains galactose and glucosamine (I.W. Sutherland, unpublished results). The lipopolysaccharide preparation did not contain any mannose, which confirms the microscopic observation that strain W93 was

non-capsulate, and further suggests that strain W93 did not produce any K antigenic polysaccharide at all. It would be of considerable value to study genetic aspects of the biosynthesis of the capsular polysaccharide using the transduction system described above, particularly since enzymatic and biosynthetic studies are already in progress on a Klebsiella (Aerobacter) aerogenes strain whose capsular polysaccharide has exactly the same chemotype as strain W70 (Ghalambor, Duckworth, Troy and Heath, 1967). The capsule of the strain used by Ghalambor et al. (1967) may well be sensitive to the polysaccharide depolymerase associated with phage PW52, and the strain may therefore be susceptible to infection (and possibly transduction) by the phage. However, the range of capsulate Klebsiella strains which can be infected by phage PW52 will probably be limited by the requirement for a suitable receptor site as well as by the specificity of the polysaccharide depolymerase, and so it may well only be possible to transduce strains of the W52/W70 group.

In preliminary experiments designed to detect joint transduction, it was found that phage PW52 grown on a str^r host was apparently capable of transducing $ade^- str^s$ recipients simultaneously to prototrophy and streptomycin resistance. However, it was then found that phage grown on a str^s donor strain had the same effect, and so an explanation involving something other than cotransduction of the two markers was sought. One possibility is that the ade^- mutants were conditionally streptomycin dependent (CSD) mutants of the type

described by Gorini and Kataja (1964). Such mutants are able to grow in the absence of their specific growth factor provided that streptomycin is present in the growth medium, and it is probable that streptomycin corrects the mutants by provoking misreading of the genetic code at the ribosomal level (streptomycin-induced phenotypic suppression: Gorini and Beckwith, 1966).

The above explanation of the behaviour of ade$^-$ mutants in transduction experiments would be confirmed if it could be shown that cells which had been treated with sub-lethal doses of streptomycin produced significant amounts of the enzyme which they were normally unable to synthesise. Further confirmation would be obtained if it could be shown that neomycin and kanamycin had a similar effect, since it has been found that these drugs are also capable of correcting a proportion of CSD mutants (Gorini and Kataja, 1965). An alternative model can be proposed in which it is assumed that the clones which appeared on Str-min.A carried a second mutation which partially suppressed the adenine requirement and simultaneously conferred on the cells the ability to grow in the presence of 1,000 µg./ml. of streptomycin (pleiotropism has frequently been observed in mutations involving the streptomycin resistance phenotype: Watanabe, 1960; Gorini and Beckwith, 1966). It would therefore be wise to study the effect of streptomycin on enzyme levels in the ade$^-$ mutants before drawing any definite conclusions, but it should be mentioned that the evidence which was obtained in the transduction experiments is clearly

not sufficient to justify a suggestion that the *ade* and *str*^{r} loci are cotransducible.

In experiments designed to detect linkage relationships between nutritional markers, the major difficulty which was encountered was that in many cases it was not possible to carry out reciprocal crosses between mutants which appeared to give significant results. This was because there was no method available which would allow high titre phage lysates to be obtained from lysogenic strains, and in order to test a sufficiently large number of mutants it was necessary to include such strains in the present study. This difficulty could be overcome by restricting analysis to non-lysogenic auxotrophic derivatives of strain W70, which could be lysogenised for use as recipients in the crosses.

The experimental procedure would be much simplified, however, if it was possible to transduce non-lysogenic bacteria directly, and this could probably be done by using antiphage antiserum in an attempt to avoid superinfection on the selection plates. Further methods of avoiding superinfection (e.g. use of ultraviolet-irradiated phage, omission from the selective medium of a factor necessary for phage adsorption) have been mentioned previously, but application of such methods would have to be preceded by a study of the characteristics of phage PW52. If it should prove possible to transduce non-lysogenic recipients, large numbers of auxotrophic mutants of strain W70 could then be isolated, tested for transducibility, and used alternately as donors and

recipients in crosses with one another. Any pairs of mutants which yielded fewer prototrophic transductants in reciprocal mutant x mutant crosses than in the corresponding mutant x wild-type crosses would suggest possible linkage relationships, and in many cases the relationships would be confirmed in donor phenotype selection tests.

However, in the present study it was found that the standard method of carrying out donor phenotype selection tests (i.e. replicating transductants from minimal agar containing the primary growth requirement of the donor strain to unsupplemented minimal agar) was unsatisfactory. Virtually all of the colonies which were replicated in this way were found to be capable of growing on the minimal agar, but it was suspected that this finding reflected either carry-over of the nutrients in the relatively large inocula or impurity of the transductant colonies on the original selective plates. The difficulties could probably be overcome by incubating the transductants on supplemented minimal medium for a shorter period of time (say 4-12 hr.) and then replicating the microcolonies which appeared to unsupplemented minimal medium. A further way of overcoming the above difficulties involves purifying the transductant clones by replating on the medium used for selection, and then testing small samples of single colonies of the resulting sublines for ability to grow on unsupplemented minimal agar. When this was done, the results strongly suggested that the thi-2 and ade-4 markers were linked, and this suggestion was supported by the finding that ade-4 (bacteria) x thi-2 (phage)

crosses yielded fewer prototrophs than ade-4 (bacteria) x wild-type (phage) crosses. Further corroborative evidence was obtained in transduction tests with other ade and thi markers, when it was found that ade markers (e.g. ade-5) which appeared to be linked to ade-4 also appeared to be linked to thi-2, whereas ade markers (e.g. ade-3) which were thought to be relatively distant from ade-4 appeared to be unlinked to thi-2.

It therefore seems highly likely that the thi-2 and ade-4 markers are closely linked and cotransducible, but definite confirmation of this would require further tests to be made. For example, it would be necessary to show that the reciprocal cross thi-2 (bacteria) x ade-4 (phage) also yielded fewer prototrophs than the thi-2 (bacteria) x wild-type (phage) cross. This could not be done with the thi-2 and ade-4 mutants themselves, because ade-4 was a derivative of strain W52 and was therefore lysogenic. However, it was found that ade-5 and thi-3 appeared to be linked in crosses where the phage was grown on the ade mutant, and since ade-4 appears to be closely linked to ade-5 and thi-2 appears to be closely linked to thi-3, this result provides partial confirmation of the ade-thi linkage relationship suggested above. It would also be necessary to demonstrate donor phenotype inheritance in the thi-2 (bacteria) x ade-4 (phage) cross, but again this could not be done with the mutants used in this study.

In the absence of information from the reciprocal cross, donor phenotype selection tests with the ade-4 and thi-2

mutants are open to at least two alternative interpretations: (1) the *thi*$^-$ transductants which were recovered had received a single fragment from the donor strain carrying the *ade*$^+$ and *thi*$^-$ alleles, or (2) the *thi*$^-$ transductants which were recovered had received a single fragment from the donor strain carrying the *thi*$^-$ allele and a suppressor of the *ade*$^-$ mutation. The suppressor mutation need not be closely linked to the *ade* locus in order to produce the *ade*$^+$ phenotype, and may or may not be associated with the *thi*$^-$ lesion in the donor strain. It may be remembered that the *thi*$^-$ donor strain was isolated following treatment with the extremely potent mutagen NTG, and so the possibility that this strain carried a second mutation (the hypothetical suppressor) is a very real one. However, the *thi*$^-$ transductant colonies were not significantly smaller on minimal A plus vitamin B1 than were the colonies of the wild-type strain, a result which suggests that they did not carry one of the more common types of suppressor mutation (see Gorini and Beckwith, 1966).

The suppressor hypothesis would be contradicted if it could be shown that transductants having the *ade*$^-$ phenotype could be recovered in *thi*-2 (bacteria) x *ade*-4 (phage) crosses, or if it could be shown that the *thi*$^-$ transductants in the *ade*-4 (bacteria) x *thi*-2 (phage) cross carried the *ade*$^+$ allele. The latter demonstration could be achieved by using *thi*$^-$ transductants as donors in a cross with the original *ade*-4 recipient strain, but it would first be necessary to isolate a non-lysogenic *thi*$^-$ transductant which would allow the phage to be

propagated on it, or to find a method of inducing the *thi*$^-$ transductants to yield high titre phage preparations. Finally, *ade*-4 x *thi*-2 crosses should yield a small proportion of *ade*$^-$*thi*$^-$ transductants, which could probably be recovered by the penicillin selection technique. Definite confirmation of the linkage relationship would then be obtained if it could be shown that *ade*$^-$*thi*$^-$ x *ade*$^+$*thi*$^+$ crosses yielded *ade*$^-$*thi*$^+$, *ade*$^+$*thi*$^-$, and *ade*$^+$*thi*$^+$ transductants.

Despite the need for confirmation as outlined above, the results of the present study are consistent with the view that the *thi*-2 and *ade*-4 markers are linked and cotransducible in *K. aerogenes* strains W52. If this should prove to be the case, it would probably be worthwhile attempting to determine which step in the purine biosynthetic pathway is blocked in the *ade*-4 mutant. That such an attempt might be of value is suggested by the finding that certain *pur* markers are linked to a *thi* marker both in *E. coli* and *S. typhimurium*. In *E. coli*, the *pur* D marker is linked to *thi*, whereas in *S. typhimurium* both *pur* D and *pur* H markers are linked to, and are cotransducible with, the *thi* marker (Taylor and Thoman, 1964; Sanderson and Demerec, 1965). It would therefore be of interest to determine whether the *ade*$^-$ mutation in *ade*-2 corresponds to the *pur* D or *pur* H mutations in *E. coli* or *S. typhimurium*. It should also be mentioned that in both *E. coli* and *S. typhimurium* several *arg* markers are closely adjacent to the *thi* and *pur* D markers, and in this context it may be significant that *arg* x *thi*-3 crosses in Klebsiella were

found to yield fewer prototrophs than arg x wild-type crosses (89/498: table 3.19; 220/1120: table 3.20). Again, however, the results of reciprocal crosses are not available, and so the possible thi-arg linkage relationship suggested by the above results must await confirmation.

From the results discussed above and in section III of this thesis, it seems likely that phage PW52 will prove useful in the study of close linkage relationships in K. aerogenes strains W52 and W70. In fact, this system may well provide as useful a method of genetic analysis as the Pseudomonas aeruginosa transduction systems, which have allowed a great many loci to be mapped in recent studies (Pearce and Loutit, 1965; Fargie and Holloway, 1965; Waltho and Holloway, 1966). However, it would be of great value to have an adequate system for mapping the broad outlines of the K. aerogenes chromosome, and this is why the conjugation experiments described in section IV of this thesis were carried out.

The major difficulty encountered in attempts to transfer the F factor from E. coli K12 to Klebsiella strains was that there was no adequate way of selecting recipients which had acquired the F factor alone, and it was therefore necessary to screen for recombinants which had acquired chromosomal markers as well as the F factor itself. On the other hand, recipients which had acquired F-lac could be selected by plating on a minimal-lactose medium, and recipients which had acquired R factors could be selected by plating on media containing appropriate drugs. However, in the experiments with F-lac

and derepressed R factors it was found that only a very low proportion of Klebsiella recipient cells acquired the factors, and it is not unreasonable to suppose that similar results would be obtained in crosses involving transfer of the F factor.

It therefore seems unlikely that it would be possible to detect chromosomal recombinants in F^+ x F^- crosses involving Klebsiella strains, particularly since genetic recombinants for any chromosomal marker occur at a frequency of only about 10^{-5} per conjugating pair in E. coli F^+ x E. coli F^- crosses (where virtually every recipient cell participates in conjugation and acquires the F factor). Indeed there may well be added difficulties in E. coli x Klebsiella crosses, in that any chromosomal material which is transferred may be rejected by the recipient or may be incapable of recombining with the recipient genome. This suggestion is supported by the finding that no chromosomal recombinants could be detected in E. coli Hfr x Klebsiella crosses, although one would expect chromosomal transfer to be initiated in any mating pairs that were formed.

The most efficient of the Klebsiella recipient strains used in this study appeared to be W107, a derivative of K. aerogenes strain A3(0)his^-str^r, but even with this strain only approximately $200/10^8$ recipient cells acquired the R_{1-14} factor from E. coli strain $J5R^+_{1-14}$ within 40 min. Strictly comparable figures are not available for crosses involving E. coli recipients, but an E. coli K12 strain described by Meynell and Datta (1967) transfers a derepressed R factor to E. coli recipients at a frequency of 2×10^{-1} per donor cell

(i.e. to roughly 5 x $10^6/10^8$ recipient cells) within 40 min. Now, in the cross described by Meynell and Datta (1967), genetic recombinants for single chromosomal markers occurred at a frequency of only about 1 per 2 x 10^5 donor cells, whereas in the E. coli $J5R^+_{1-14}$ x W107 cross only about 1 x 10^4 donor cells transferred the R factor at all. It therefore seems unlikely that chromosomal transfer would be detected in $J5R^+_{1-14}$ x Klebsiella crosses, and even more unlikely in Klebsiella x Klebsiella crosses where the level of R_{1-14} transfer was found to be lower than in $J5R^+_{1-14}$ x Klebsiella crosses.

As mentioned previously, the results of experiments involving transfer of R factors suggest that the Klebsiella strains used in this study are poor donors in crosses with E. coli or Klebsiella recipients. This suggestion is supported by the finding of de Haan et al. (1963) that the F'13 episome could not be transferred from an F' Klebsiella strain to E. coli or Klebsiella recipients, and by the finding of Guinée, Scholtens and Willens (1967) that transfer of an R factor from a Klebsiella strain to Salmonella panama recipients rarely succeeded. Some of the Klebsiella donors used in this study were capsulate and others were non-capsulate, but there was no indication that the non-capsulate donors were any more efficient than the capsulate ones. This result was rather surprising, because it was suspected that capsules might well occlude the specialised pili which are thought to be associated with conjugal transfer of genetic material (Brinton, 1965;

Meynell and Datta, 1967). However, under normal conditions in broth culture the Klebsiella strains used in this study produce capsules which usually extend to less than 3 μ beyond the cell surface, whereas F pili can be over 20 μ in length (Lawn, 1966).

A more likely explanation for the behaviour of R^+ Klebsiella donors would seem to be that only a very small proportion of the cells which carry an R factor are capable of synthesising specialised pili, even when the R factor is one which is derepressed in an E. coli host cell. No direct evidence was obtained to support this suggestion, but there seem to be reasonable grounds for considering it since it was found that only a very few (if any) of the cells in an R^+_{1-14} Klebsiella culture were capable of adsorbing the male-specific phage MS-2. This finding may indicate that the phage MS-2 receptor sites on the surfaces of specialised F-type pili are somehow occluded even in non-capsulate Klebsiellas, or may merely reflect a reduced ability of genetic material derived from a "foreign" host to function in Klebsiella cells. An alternative possibility is that the Klebsiella strains used in this study carry an endogenous repressor of the conjugation functions of the R_{1-14} factor; one way of testing this hypothesis would be to study the behaviour in Klebsiella cells of a mutant R factor whose conjugation functions were no longer sensitive to the repressor which the R factor normally produces on its own account (i.e. a factor carrying a mutation analagous to o^c mutations in the lactose regulatory system of E. coli:

Jacob and Monod, 1961). However, despite these observations it seems highly likely that some of the cells in an R^+ Klebsiella culture do synthesise functional specialised pili, because transfer of the R factor could almost always be detected and no evidence was obtained to suggest that this transfer did not occur by the normal mechanisms of conjugation.

A further conclusion which was drawn from the results of the experiments involving transfer of F-lac and R factors was that the Klebsiella strains used in this study were poor recipients in crosses with E. coli or Klebsiella donors. The most efficient mating was found to occur on membrane filters or on the surface of a solid medium, but even so the frequency of transfer was never more than about 1×10^{-6} per donor cell. These findings are difficult to assess, however, because very little is known about the factors which determine recipient ability even in the intensively studied E. coli K12 mating system. One factor which does not seem to be of great importance in Klebsiella systems is capsulation - certain capsulate and non-capsulate strains were found to be equally efficient as recipients in crosses with the R^+ strain K66. This result is rather surprising, because it is difficult to understand how an F-type pilus can penetrate the capsule of a recipient bacterium to reach the underlying cell wall. It seems possible that recipient cultures of a capsulate strain contain a few genetically non-capsulate cells which are the only ones to acquire the R factor, but no evidence was found to support this hypothesis, and indeed the fact that the R^+

clones recovered from such a culture were always found to be genetically capsulate tends to contradict it. A more likely explanation is that the donor pilus establishes contact with the recipient cell at a time when the continuity of the capsule is temporarily broken, for example during cell division, and if this is so the reformed capsule may even serve to stabilise the resulting mating pair. However, it may be significant that the strain which appeared to act as the most efficient recipient was W107, a derivative of *K. aerogenes* A3(0)*his*$^{-}$*str*^{r} which formed flatter, less smooth, colonies than the parent non-capsulate organism.

It seems likely that other Klebsiella strains may be capable of acting as more efficient recipients than W107. This possibility is suggested by the finding of Hamon (1956) that a genetic determinant of colicinogeny could be transferred from *E. coli* strain K30 to two *K. pneumoniae* strains. This finding has not been confirmed, but since the transfer system did not appear to require a powerful method of selecting *col*$^{+}$ recipients it would seem that the Klebsiella strains were capable of acting as extremely efficient recipients. Further relevant evidence was recently obtained by Stouthamer and Tieze (1966), when they found in preliminary experiments that several of their Klebsiella strains could transfer the bacteriocinogenic property to non-bacteriocinogenic Klebsiella strains. However, these experiments were not described in any detail and have not yet been confirmed.

In conclusion, it may be worth recalling that *Salmonella*

typhimurium strain LT-2 has been shown to be a poor recipient of certain R factors (Watanabe and Fukasawa, 1961a; section IV of this thesis). Nevertheless, conjugation studies have provided a considerable amount of information about this organism, and in fact the results of F-mediated and col-mediated transfer studies have been combined with the results of fine structure transduction studies to yield a detailed map of the whole chromosome (Sanderson and Demerec, 1965). The results so far obtained with K. aerogenes strain A3 are not therefore too discouraging, and suitable conditions allowing chromosomal transfer to or from this strain may yet be found. If not, other Klebsiella strains may prove to be extremely fertile when infected with one of the wide array of conjugation factors which have now been discovered, and indeed Smith and Armour (1966) have recently mentioned Klebsiella strains which are capable of transferring R factors to E. coli recipients at frequencies of up to 10^{-2} per donor cell. In any event, the results of the present study suggest that there is now a transduction system available which promises to allow detailed analysis of short regions of the Klebsiella chromosome.

SUMMARY

(1) Numerous attempts were made to detect transformation of Klebsiella strains. In these experiments, DNA was extracted from donor bacteria by a variety of techniques, and the resulting preparations were applied to genetically marked recipient bacteria. In some cases the recipient cells had previously been treated with agents which were known to affect the integrity of the cell wall, and in other cases the recipient cells were taken from ordinary broth cultures. No evidence of transformation was obtained in any experiment; possible reasons for this finding are discussed.

(2) Attempts were made to demonstrate lysogeny in Klebsiella aerogenes strain A3, but no positive results were obtained. Similarly, none of the other Klebsiella strains tested were found to release phages active on K. aerogenes strain A3.

(3) Several bacteriophages were isolated from natural sources by the enrichment culture technique, with K. aerogenes strain A3 as host. Five of the phages were found to produce turbid zones of partial clearing when plated on strain A3, but none were found to be capable of lysogenising cells of strain A3.

(4) Attempts were made to transduce K. aerogenes strain A3 and genetically marked derivatives of this strain with a number of virulent phages. Several different methods of minimising superinfection by free phage were used, but no transductants were recovered in any experiment.

(5) Auxotrophic mutants of lysogenic and sensitive indicator Klebsiella strains were prepared, and used in preliminary mixed cultivation experiments. The results suggested that genetic exchange had occurred in tests involving K. aerogenes strain W52.

(6) K. aerogenes strain W52 was found to be lysogenic, but did not appear to be ultraviolet inducible. However, cultures of strain W52 which had been treated with the potent mutagen N-methyl-N'-nitro-N-nitrosoguanidine were found to lyse and release large numbers of plaque-forming particles. This result suggests that NTG is an effective inducing agent, but the possibility that phage lysates obtained by NTG-induction would be unsuitable for use in transduction experiments is discussed.

(7) The phage released by strain W52 was propagated in cells of a sensitive strain and designated PW52. Preliminary spot tests suggested that phage PW52 was capable of mediating transduction, with the important limitation that transductants were only recovered if the recipient bacteria were immune to lytic infection by the phage.

(8) The characteristics of the system of genetic exchange which appeared to involve phage PW52 were studied, and although absolute confirmation was not obtained the results strongly suggested that phage PW52 was in fact capable of mediating transduction at frequencies varying from 5×10^{-7} to 3×10^{-9} per phage particle adsorbed. No evidence was found to suggest that any of the other known mechanisms of

genetic exchange were involved in the system which was being studied.

(9) It was found that non-mucoid mutants of K. aerogenes strains W52 and W70 could not be transduced with respect to any of the markers tested, and a possible reason for this finding is suggested.

(10) Experiments were carried out to test for joint transduction of streptomycin resistance and maltose utilisation markers, but no positive results were obtained.

(11) In further preliminary attempts to detect linkage of genetic markers, it was found that certain ade markers appeared to be cotransducible with a str^r marker, but later experiments suggested that this result could be explained without postulating a specific joint transduction of the markers concerned.

(12) Some 22 auxotrophic mutants of K. aerogenes strains W52 and W70 were isolated following treatment with N-methyl-N'-nitro-N-nitrosoguanidine, and used in a large series of transduction tests. The results of these experiments suggested several linkage relationships, which are discussed in some detail. One of the possible linkage relationships was partially confirmed by the donor phenotype selection technique, and several tests which might be expected to provide further confirmation are outlined.

(13) Mixed cultivation experiments with auxotrophic derivatives of K. aerogenes strain A3 and K. pneumoniae strain 1.9 failed to yield any evidence of genetic recombination.

(14) Transfer of the F-lac episome from an E. coli K12

strain to a *lac*$^-$ mutant of *K. aerogenes* strain A3 was observed, but the F-*lac*$^+$ recombinants were not found capable of transferring the episome to an F$^-$*lac*$^-$ strain of *E. coli*.

(15) Attempts to detect transfer of the F factor from *E. coli* K12 to Klebsiella strains, or of chromosomal material from an Hfr strain of *E. coli* K12 to Klebsiella strains, were unsuccessful.

(16) Attempts were made to demonstrate elimination of the genetic determinant of bacteriocinogeny from *K. pneumoniae* strain 1.2 by treatment with acridine dyes, but the results were negative. Experiments designed to detect transfer of the bacteriocin determinant from strain 1.2 to other Klebsiella strains failed to yield any evidence that the determinant was transmissible from cell to cell.

(17) It was found that infectious drug resistance (R) factors could be transferred between Klebsiella strains, but transfer was always found to occur at low frequency. The mechanism of the genetic exchange was not characterised fully, but appeared to be a typical example of bacterial conjugation.

(18) Experiments involving transfer of an R factor from a capsulate donor strain to capsulate and non-capsulate recipient strains were carried out. No evidence was found to suggest that the non-capsulate strains were capable of acquiring the R factor more efficiently than the capsulate strain.

(19) The behaviour of Klebsiella strains in high-frequency resistance transfer systems was studied, and it was

concluded that the strains used were not capable of acting as efficient recipients of R factors, and were probably poor donors as well.

(20) An R factor which was no longer sensitive to a repressor of its conjugation functions was transferred to Klebsiella strains, and the R^+ strains so obtained were used in attempts to detect transfer of chromosomal genetic material to an R^- Klebsiella strain. The R^- strain which was used had been found to act as a reasonably efficient recipient of the derepressed R factor alone, but no chromosomal recombinants were recovered.

(21) Derivatives of K. aerogenes strain A3 carrying a derepressed R factor were found to be insensitive to the male-specific bacteriophage MS-2, and appeared to be unable to adsorb significant numbers of MS-2 particles. In control experiments, an E. coli K12 strain carrying the same factor was found to be sensitive to phage MS-2 and capable of removing some 75% of the MS-2 particles from a culture supernatant.

BIBLIOGRAPHY

Adams, J.N. and Luria, S.E. (1958). Proc. natn. Acad. Sci. U.S.A., 44, 590.

Adams, M.H. (1959). Bacteriophages. New York: Interscience.

Adams, M.H. and Park, B.H. (1956). Virology, 2, 719.

Adelberg, E.A., Mandel, M. and Chen, G.C.C. (1965). Biochem. biophys. Res. Commun., 18, 788.

Allan, R.K. and McCalla, D.R. (1966). Can. J. Microbiol., 12, 202.

Amati, P. (1962). J. Bact., 83, 433.

Anagnostopolous, C. and Crawford, I.P. (1961). Proc. natn. Acad. Sci. U.S.A., 47, 378.

Anagnostopolous, C. and Spizizen, J. (1961). J. Bact., 81, 741.

Atchley, W.A. (1951). Cold Spring Harb. Symp. quant. Biol., 16, 413.

Avery, O.T., MacLeod, C.M. and McCarty, M. (1944). J. exp. Med., 79, 137.

Balassa, G. (1963). Bact. Rev., 27, 228.

Balows, A., Weaver, R.H. and Humphries, J.C. (1955). J. Bact., 69, 139.

Baron, L.S. (1963). In Methodology in Basic Genetics (ed. Burdette, W.J.). San Francisco: Holden-Day Inc.

Baron, L.S. (1965). Trans. N.Y. Acad. Sci., 27, 999.

Baron, L.S., Carey, W.F. and Spilman, W.M. (1959). Proc. natn. Acad. Sci. U.S.A., 45, 1752.

Baron, L.S., Spilman, W.M. and Carey, W.F. (1959). Science, N.Y., 130, 566.

Belser, W.M. and Bunting, M. (1956). J. Bact., 72, 582.

Bernstein, H.L. (1958). Symp. Soc. exp. Biol., 12, 93.

Bertani, G. (1951). J. Bact., 62, 293.

Bhaskaran, K. (1960). J. gen. Microbiol., 23, 47.

Bhaskaran, K. (1964). Bull. Wld Hlth Org., 30, 845.

Boivin, A. (1947). Cold Spring Harb. Symp. quant. Biol., 12, 7.

Boivin, A., Vandrely, R. and Lehoult, Y. (1945). C. r. hebd. Séanc. Acad. Sci., Paris, 221, 646.

Borman, E.K., Stuart, C.A. and Wheeler, K.M. (1944). J. Bact., 48, 351.

Boyer, H. (1964). J. Bact., 88, 1652.

Breed, R.S., Murray, E.G.D. and Smith, N.R. (1957). Bergey's Manual of Determinative Bacteriology, 7th edition. London: Bailliere, Tindall and Cox, Ltd.

Brinton, C.C. (1965). Trans. N.Y. Acad. Sci., 27, 1003.

Burton, K. (1956). Biochem. J., 62, 315.

Cairns, J. (1963). Cold Spring Harb. Symp. quant. Biol., 28, 43.

Campbell, A. (1964). In The Bacteria, volume 5 (ed. Gunsalus, I.C. and Stanier, R.Y.). New York and London: Academic Press.

Carere, A. and Spada-Sermonti, I. (1964). J. Bact., 88, 226.

Cavalli-Sforza, L.L. (1950). Boll. Ist. sieroter. milan., 29, 281.

Cavalli-Sforza, L.L., Lederberg, J. and Lederberg, E.M. (1953). J. gen. Microbiol., 8, 89.

Centifanto, Y.M. and Silver, W.S. (1964). J. Bact., 88, 776.

Chargaff, E., Schulman, H.M. and Shapiro, H.S. (1957). Nature, Lond., 180, 851.

Ciuca, M., Eustatziou, S., Barber, C., Voinea, M. and Tulpan, G. (1959). Archs roum. Path. exp. Microbiol., 19, 367.

Clark, A.J. and Margulies, A.D. (1965). Proc. natn. Acad. Sci. U.S.A., 53, 451.

Clarke, C.H. (1960). Genetic Studies with Salmonella and Klebsiella, Ph.D. Thesis, Birmingham University.

Clarke, C.H. (1961). Nature, Lond., 190, 194.

Clarke, C.H. (1964). Experientia, 20, 83.

Clarke, C.H. and MacPhee, D.G. (1965). Experientia, 21, 18.

Clowes, R.C. (1958). J. gen. Microbiol., 18, 154.

Clowes, R.C. (1951). Nature, Lond., 190, 988.

Clowes, R.C. (1963). Genet. Res., 4, 162.

Clowes, R.C. (1965). Zentbl. Bakt. ParasitKde., Abt. 1 Orig., 196, 152.

Coetzee, J.N. and Sacks, T.G. (1960). J. gen. Microbiol., 23, 445.

Colwell, R.R. and Mandel, M. (1964). J. Bact., 87, 1412.

Conrad, H.E., Bamburg, J.R., Epley, J.D. and Kindt, T.J. (1966). Biochemistry, 5, 2808.

Cowan, S.T., Steel, K.J., Shaw, C. and Duguid, J.P. (1960). J. gen. Microbiol., 23, 601.

Crawford, E.M. and Gesteland, R.F. (1964). Virology, 22, 165.

Darrell, J.H. and Hurdle, A.D.F. (1964). J. clin. Path., 17, 617.

Datta, N. (1965). Br. med. Bull., 21, 254.

Datta, N., Lawn, A.M. and Meynell, E. (1966). J. gen. Microbiol., 45, 365.

Davis, B.D. (1950). J. Bact., 60, 507.

Davis, B.D. and Mingioli, E.S. (1950). J. Bact., 60, 17.

Dawson, M.H. and Sia, R.H.P. (1931). J. exp. Med., 54, 681.

Demerec, M., Blomstrand, I. and Demerec, Z.E. (1955). Proc. natn. Acad. Sci. U.S.A., 41, 359.

Demerec, M. and Hartman, P.E. (1959). A. Rev. Microbiol., 13, 377.

Dettori, R., Maccacaro, G.A. and Piccinin, G.L. (1961). G. Microbiol., 9, 141.

Dettori, R., Maccacaro, G.A. and Turri, M. (1963). G. Microbiol., 11, 15.

Driskell-Zamenhof, P. (1964). In The Bacteria, volume 5 (ed. Gunsalus, I.C. and Stanier, R.Y.). New York and London: Academic Press.

Duguid, J.P. (1951). J. Path. Bact., 63, 673.

Durlakowa, I., Maresz-Babczyszyn, J., Przondo-Hessek, A., Lusar, Z. and Mroz-Kurpiela, E. (1964a). Archvm Immun. Ther. Exp., 12, 308.

Durlakowa, I., Maresz-Babczyszyn, J., Przondo-Hessek, A., Lusar, Z. and Mroz-Kurpiela, E. (1964b). Archvm Immun. Ther. Exp., 12, 319.

Dushman, M.B. (1963). J. Bact., 86, 1173.

Dussoix, D. and Arber, W. (1962). J. molec. Biol., 5, 37.

Edwards, P.R. (1929). J. Bact., 17, 339.

Edwards, P.R. and Ewing, W.H. (1955). Identification of Enterobacteriaceae, Minneapolis: Burgess Pub. Co.

Edwards, P.R. and Fife, M.A. (1952). J. infect. Dis., 91, 92.

Eustatziou, S., Barber, C., Voinea, M. and Tulpan, G. (1960). Archs roum. Path. exp. Microbiol., 19, 367.

Eustatziou, S., Eustatziou, G., Antohi, M., Horodniceanu, T., Rusu, V. and Alexandresco, A. (1962). Archs roum. Path. exp. Microbiol., 21, 131.

Falkow, S. and Baron, L.S. (1962). J. Bact., 84, 581.

Fargie, B. and Holloway, B.W. (1965). Genet. Res., 6, 284.

Farkas-Himsley, H. and Seyfried, P.L. (1963). Can. J. Microbiol., 9, 329.

Frédéricq, P. (1954). C. r. Séanc. Soc. Biol., 148, 746.

Frédéricq, P. (1957). A. Rev. Microbiol., 11, 7.

Frédéricq, P. (1958). Symp. Soc. exp. Biol., 12, 104.

Frédéricq, P. (1965). Zentbl. Bakt. ParasitKde., Abt. 1 Orig., 196, 142.

Fox, M.S. (1962). Proc. natn. Acad. Sci. U.S.A., 48, 1043.

Gebicki, J.M. and James, A.M. (1958). Nature, Lond., 182, 725.

Ghalambor, M.A., Duckworth, D.H., Troy, F.A. and Heath, E.C. (1967). Fedn Proc. Fedn Am. Socs exp. Biol., p. 346, no. 509.

Glover, S.W. (1962). Genet. Res., 3, 448.

Goldschmidt, E.P. and Landman, O.E. (1962). J. Bact., 83, 690.

Gorini, L. and Beckwith, J.R. (1966). A. Rev. Microbiol., 20, 401.

Gorini, L. and Kataja, E. (1964). Proc. natn. Acad. Sci. U.S.A., 51, 487.

Gorini, L. and Kataja, E. (1965). Biochem. biophys. Res. Commun., 18, 656.

Gorini, L. and Kaufman, H. (1960). Science, N.Y., 131, 604.

Griffith, F. (1928). J. Hyg., Camb., 27, 113.

Gross, J.D. (1964). In The Bacteria, volume 5 (ed. Gunsalus, I.C. and Stanier, R.Y.). New York and London: Academic Press.

Gross, J.D. (1965). Br. med. Bull., 21, 206.

Gross, J.D. and Caro, L. (1965). Science, N.Y., 150, 1679.

Guinée, P.A.M., Scholtens, R.Th. and Willems, H.M.C.C. (1967). Antonie van Leeuwenhoek, 33, 30.

Gunderson, W.B., Jyssum, K. and Lie, S. (1962). J. Bact., 83, 616.

Gwinn, D.D. and Thorne, C.B. (1964). J. Bact., 87, 519.

Haan, P.G. de, Stouthamer, A.H., Felix, H.S. and Mol, A.K. (1963). Antonie van Leeuwenhoek, 29, 407.

Hadley, P. (1925). Proc. Soc. exp. Biol. Med., 23, 109.

Hamilton-Miller, J.M.T. (1965). Biochem. biophys. Res. Commun., 20, 688.

Hamilton-Miller, J.M.T. (1966). Biochem. J., 100, 675.

Hamon, Y. (1956). C. r. hebd. Séanc. Acad. Sci., Paris, 242, 2064.

Hamon, Y. and Peron, Y. (1963). C. r. hebd. Acad. Sci., Paris, 257, 309.

Harada, K., Suzuki, M., Kameda, M. and Mitsuhashi, S. (1960). Jap. J. exp. Med., 30, 289.

Hardy, P.H. and Nell, E.E. (1967). Nature, Lond., 214, 414.

Hartman, P.E. (1963). In Methodology in Basic Genetics (ed. Burdette, W.J.). San Francisco: Holden-Day Inc.

Hartman, P.E. and Goodgal, S.H. (1959). A. Rev. Microbiol., 13, 465.

Hartman, P.E., Loper, J.C. and Serman, D. (1960). J. gen. Microbiol., 22, 323.

Hayes, W. (1953). Cold Spring Harb. Symp. quant. Biol., 18, 75.

Hayes, W. (1957). J. gen. Microbiol., 16, 97.

Hayes, W. (1964). The Genetics of Bacteria and their Viruses, Oxford: Blackwell.

Hayes, W. (1966a). Proc. R. Soc., Series B, 164, 230.

Hayes, W. (1966b). Proc. R. Soc., Series B, 165, 1.

Henriksen, S.D. (1954). Acta path. microbiol. scand., 34, 266.

Heumann, W. (1962). Z. VererbLehre., 93, 441.

Heyl, J.G. (1957). Antonie van Leeuwenhoek, 23, 33.

Hirota, Y. (1960). Proc. Natn. Acad. Sci. U.S.A., 46, 57.

Hoffmann-Berling, H. and Maze, R. (1964). Virology, 22, 305.

Hofschneider, P.H. and Preuss, A. (1963). J. molec. Biol., 7, 450.

Holloway, B.W. (1955). J. gen. Microbiol., 13, 572.

Holloway, B.W. and Fargie, B. (1960). J. Bact., 80, 362.

Horiuchi, K. and Adelberg, E.A. (1965). J. Bact., 89, 1231.

Hormaeche, E. (1958). Int. Bull. bact. Nomencl. Taxon., 8, 25.

Hormaeche, E. and Edwards, P.R. (1958). Int. Bull. bact. Nomencl. Taxon., 8, 111.

Hormaeche, E. and Edwards, P.R. (1960). Int. Bull. bact. Nomencl. Taxon., 10, 71.

Hotchkiss, R.D. (1951). Cold Spring Harb. Symp. quant. Biol., 16, 457.

Hotchkiss, R.D. (1954). Proc. natn. Acad. Sci. U.S.A., 40, 49.

Humphries, J.C. (1948). J. Bact., 56, 683.

Ishibashi, M. (1967). J. Bact., 93, 379.

Jackson, S. (1962). Br. med. Bull., 18, 24.

Jacob, F. (1966). Science, N.Y., 152, 1470.

Jacob, F., Brenner, S. and Cuzin, F. (1963). Cold Spring Harb. Symp. quant. Biol., 28, 329.

Jacob, F. and Monod, J. (1961). J. molec. Biol., 3, 318.

Jacob, F., Schaeffer, P. and Wollman, E.L. (1960). Symp. Soc. gen. Microbiol., 10, 67.

Jacob, F. and Wollman, E.L. (1958). C. r. hebd. Séanc. Acad. Sci., Paris, 247, 154.

Jacob, F. and Wollman, E.L. (1959). In The Viruses, volume 2 (ed. Burnet, F.M. and Stanley, W.M.). New York and London: Academic Press.

Jacob, F. and Wollman, E.L. (1961). Sexuality and the Genetics of Bacteria. New York and London: Academic Press.

Julianelle, L.A. (1926). J. exp. Med., 44, 113.

Kaiser, A.D. and Hogness, D.S. (1960). J. molec. Biol., 2, 392.

Kasuya, M. (1964). J. Bact., 88, 322.

Kauffmann, F. (1954). Enterobacteriaceae, 2nd edition. Munksgaard: Copenhagen.

Kauffmann, F. (1966). The Bacteriology of Enterobacteriaceae, 3rd edition. Munksgaard: Copenhagen.

Keller, R. and Olds, M.J. (1958). Bact. Proc., p. 57, G104.

Kelly, M.S. and Pritchard, R.H. (1965). J. Bact., 89, 1314.

Knolle, P. (1967). Biochem. biophys. Res. Commun., 27, 81.

Kohn, A. (1960). J. Bact., 79, 697.

Krieg, R.E. and Lockhart, W.R. (1966). J. Bact., 92, 1275.

Lawn, A.M. (1966). J. gen. Microbiol., 45, 377.

Lederberg, J. (1950). Meth. med. Res., 3, 5.

Lederberg, J. (1952). Physiol. Rev., 32, 403.

Lederberg, J. and Lederberg, E.M. (1952). J. Bact., 63, 399.

Lederberg, J., Lederberg, E.M., Zinder, N.D. and Lively, E.R. (1951). Cold Spring Harb. Symp. quant. Biol., 16, 413.

Lederberg, J. and St. Clair, J. (1958). J. Bact., 75, 143.

Lederberg, J. and Tatum, E.L. (1946). Nature, Lond., 158, 558; and Cold Spring Harb. Symp. quant. Biol., 11, 113.

Leive, L. (1965a). Biochem. biophys. Res. Commun., 18, 13.

Leive, L. (1965b). Proc. natn. Acad. Sci. U.S.A., 53, 745.

Lennox, E.S. (1955). Virology, 1, 190.

Loeb, T. (1960). Science, N.Y., 131, 932.

Loeb, T. and Zinder, N.D. (1961). Proc. natn. Acad. Sci. U.S.A., 47, 282.

Loutit, J.S. (1958). J. gen. Microbiol., 18, 315.

Loveless, A. and Howarth, S. (1959). Nature, Lond., 184, 1780.

Luria, S.E. and Burrous, J.W. (1957). J. Bact., 74, 461.

Luria, S.E. and Delbruck, M. (1943). Archs Biochem., 1, 207.

Lwoff, A., Siminivotch, L. and Kjeldgaard, N. (1950). C. r. hebd. Séanc. Acad. Sci., Paris, 231, 190.

Maccacaro, G.A. (1955). Nature, Lond., 176, 125.

Maccacaro, G.A. and Comolli, R. (1956). J. gen. Microbiol., 15, 121.

Mahl, M.C., Wilson, P.W., Fife, M.A. and Ewing, W.H. (1965). J. Bact., 89, 1482.

Makela, P.H., Lederberg, J. and Lederberg, E.M. (1962). Genetics, 47, 1427.

Mandel, M. and Rownd, R. (1964). In Taxonomic Biochemistry and Serology (ed. C.A. Leone). New York: Ronald Press.

Marmur, J. (1961). J. molec. Biol., 3, 208.

Marmur, J., Falkow, S. and Mandel, M. (1963). A. Rev. Microbiol., 17, 329.

Martin, G. and Jacob, F. (1962). C. r. hebd. Séanc. Acad. Sci., Paris, 254, 3589.

Matney, T.S. and Achenbach, N.E. (1962). J. Bact., 84, 874.

Matsushiro, A. (1963). Virology, 19, 475.

McCarty, M., Taylor, H.E. and Avery, O.T. (1946). Cold Spring Harb. Symp. quant. Biol., 11, 177.

McQuillen, K. (1958). Biochim. biophys. Acta, 27, 410.

McQuillen, K. (1960). In The Bacteria, volume 1 (ed. Gunsalus, I.C. and Stanier, R.Y.). New York and London: Academic Press.

Meynell, E. and Datta, N. (1966a). Genet. Res., 7, 134.

Meynell, E. and Datta, N. (1966b). Genet. Res., 7, 141.

Meynell, E. and Datta, N. (1967). Nature, Lond., 214, 885.

Meynell, G.G. and Lawn, A.M. (1967). Genet. Res., 9, 359.

Milch, H. and Deak, S. (1964-65). Acta microbiol. hung., 11, 251.

Miles, A.A. and Misra, S.S. (1938). J. hyg., Camb., 38, 732.

Mitchell, P. and Moyle, J. (1956). Nature, Lond., 178, 993.

Mitsuhashi, S., Harada, K. and Hashimoto, H. (1960). Jap. J. exp. Med., 30, 179.

Mitsuhashi, S., Hashimoto, H., Egawa, R., Tanaka, T. and Nagai, Y. (1967). J. Bact., 93, 1242.

Miyake, T. and Demerec, M. (1959). Nature, Lond., 183, 1586.

Mohan, R.R., Kronish, D.P., Pianotti, R.S., Lepstein, R. and Schwartz, B.S. (1965). J. Bact., 90, 1355.

Molina, A.M. (1964). G. Microbiol., 12, 107.

Molina, A.M., Calegari, L. and Monti-Bragadin, C. (1965). G. Microbiol., 13, 123.

Monk, M. and Clowes, R.C. (1964). J. gen. Microbiol., 36, 385.

Morse, M.L. (1954). Genetics, 39, 984.

Morse, M.L. (1959). Proc. natn. Acad. Sci. U.S.A., 45, 722.

Morse, M.L., Lederberg, E.M. and Lederberg, J. (1956). Genetics, 41, 142.

Muecke, D., Bauer, R. and Brock, J. (1966). Acta biol. med. germ., 17, 547.

Nakaya, R., Nakamura, A. and Murata, Y. (1960). Biochem. biophys. Res. Commun., 3, 654.

Novick, R.P. (1963). J. gen. Microbiol., 33, 121.

Ørskov, F. and Ørskov, I. (1961). Acta path. microbiol. scand., 51, 280.

Ørskov, F., Ørskov, I. and Kauffmann, F. (1961). Acta path. microbiol. scand., 51, 291.

Ørskov, I. (1954). Acta path. microbiol. scand., 34, 145.

Ørskov, I. and Ørskov, F. (1960). Acta path. microbiol. scand., 48, 37.

Ørskov, I. and Ørskov, F. (1966). J. Bact., 91, 69.

Ozeki, H. (1956). Carn. Inst. Wash. Publ., 612, 97.

Ozeki, H. (1959). Genetics, 44, 457.

Ozeki, H. and Howarth, S. (1961). Nature, Lond., 190, 986.

Pakula, R. (1965). Can. J. Microbiol., 11, 811.

Pakula, R. and Walczak, W. (1963). J. gen. Microbiol., 31, 125.

Park, B.H. (1956). Virology, 2, 711.

Parr, L.W. (1939). Bact. Rev., 3, 1.

Pearce, L.E. and Loutit, J.S. (1965). J. Bact., 89, 58.

Prozesky, O.W. and Coetzee, J.N. (1966). Nature, Lond., 209, 1262.

Ptashne, M. (1965). J. molec. Biol., 11, 829.

Rakieten, M.L., Eggerth, A.H. and Rakieten, T.L. (1940). J. Bact., 40, 529.

Ravin, A.W. (1957). Ann. N.Y. Acad. Sci., 68, 335.

Ravin, A.W. (1958). A. Rev. Microbiol., 12, 309.

Ravin, A.W. (1961). Adv. Genet., 10, 61.

Reeves, P. (1965). Bact. Rev., 29, 24.

Repaske, R. (1958). Biochim. biophys. Acta, 30, 225.

Richter, A. (1961). Genet. Res., 2, 333.

Rolfe, B. and Holloway, B.W. (1966). J. Bact., 92, 43.

Rothberg, N.W. and Swartz, M.N. (1965). J. Bact., 90, 294.

Sanborn, J.R. (1944). J. Bact., 48, 211.

Sanderson, K.E. and Demerec, M. (1965). Genetics, 51, 897.

Sandford, P.A. and Conrad, H.E. (1966). Biochemistry, 5, 1508.

Schaeffer, P. (1964). In The Bacteria, volume 5 (ed. Gunsalus, I.C. and Stanier, R.Y.). New York and London: Academic Press.

Schlissel, H.J. and Sword, C.P. (1966). J. Bact., 92, 1357.

Sherman, J.M. and Wing, H.U. (1937). J. Bact., 33, 315.

Signer, E.R. and Beckwith, J.R. (1966). J. molec. Biol., 22, 33.

Smith, D.H. (1966). New Engl. J. Med., 275, 625.

Smith, D.H. and Armour, S.E. (1966). Lancet, 2, 15.

Smith, S.M. and Stocker, B.A.D. (1962). Br. med. Bull., 18, 46.

Sneath, P.H.A. and Lederberg, J. (1961). Proc. natn. Acad. Sci. U.S.A., 47, 86.

Sparling, P.F. (1966). J. Bact., 92, 1364.

Spizizen, J., Reilly, B.E. and Evans, A.H. (1966). A. Rev. Microbiol., 20, 371.

Stamatin, N. (1959). Annls. Inst. Pasteur, Paris, 96, 502.

Starlinger, P. (1958). Z. Naturf., 13 B, 489.

Stocker, B.A.D., Smith, S.M. and Ozeki, H. (1963). J. gen. Microbiol., 30, 201.

Stocker, B.A.D., Zinder, N.D. and Lederberg, J. (1953). J. gen. Microbiol., 9, 410.

Stonehill, E.H. and Hutchison, D.J. (1966). J. Bact., 92, 136.

Stouthamer, A.H. and Tieze, G.A. (1966). Antonie van Leeuwenhoek, 32, 171.

Sugino, Y. and Hirota, Y. (1962). J. Bact., 84, 902.

Sutherland, I.W. (1958). Honours Dissertation, University of Edinburgh.

Sutherland, I.W. and Wilkinson, J.F. (1965). J. gen. Microbiol., 39, 373.

Tatum, E.L. and Lederberg, J. (1947). J. Bact., 53, 673.

Taylor, A.L. and Thoman, M.S. (1964). Genetics, 50, 659.

Taylor, M. and Yanofsky, C. (1964). Biochem. biophys. Res. Commun., 17, 798.

Thorne, C.B. (1962). J. Bact., 83, 106.

Tomasz, A. (1965). Nature, Lond., 208, 155.

Tomasz, A. and Hotchkiss, R.D. (1964). Proc. natn. Acad. Sci. U.S.A., 51, 480.

Tomasz, A. and Mosser, J.L. (1966). Proc. natn. Acad. Sci. U.S.A., 55, 58.

Turri, M. and Maccacaro, G.A. (1960). G. microbiol., 8, 1.

Wacker, A. and Laschet, L. (1960). Arzneimittel-Forsch., 10, 488.

Waltho, J.A. and Holloway, B.W. (1966). J. Bact., 92, 35.

Watanabe, T. (1960). J. gen. Microbiol., 22, 102.

Watanabe, T. (1963a). Bact. Rev., 27, 87.

Watanabe, T. (1963b). J. Bact., 85, 788.

Watanabe, T. (1964). Meth. med. Res., 10, 202.

Watanabe, T. (1966). New Engl. J. Med., 275, 888.

Watanabe, T. and Fukasawa, T. (1961a). J. Bact., 81, 669.

Watanabe, T. and Fukasawa, T. (1961b). J. Bact., 81, 679.

Watanabe, T., Nishida, H., Ogata, C., Arai, S. and Sato, S. (1964). J. Bact., 88, 716.

Watanabe, T. and Watanabe, M. (1959). J. gen. Micriobiol., 21, 16.

Wilkinson, J.F. (1958). Bact. Rev., 22, 46.

Wollman, E.L., Jacob, F. and Hayes, W. (1956). Cold Spring Harb. Symp. quant. Biol., 21, 141.

Yanofsky, C. and Lennox, E.S. (1959). Virology, 8, 425.

Yoshikawa, H. and Sueoka, N. (1963). Proc. natn. Acad. Sci. U.S.A., 49, 559.

Young, F.E. and Spizizen, J. (1961). J. Bact., 81, 823.

Zinder, N.D. (1955). J. cell. comp. Physiol., 45 (suppl. 2), 23.

Zinder, N.D. (1958). Virology, 5, 291.

Zinder, N.D. (1960a). Science, N.Y., 131, 813.

Zinder, N.D. (1960b). Science, N.Y., 131, 924.

Zinder, N.D. (1963). In Methodology in Basic Genetics (ed. Burdette, W.J.). San Francisco: Holden-Day, Inc.

Zinder, N.D. and Lederberg, J. (1952). J. Bact., 64, 679.

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